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Central Composite Design Implemented Azilsartan Medoxomil Loaded Nanoemulsion to Improve Its Aqueous Solubility and Intestinal Permeability: In

Vitro and Ex Vivo Evaluation

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Abstract

The present research attempted to design and develop a nanoemulsion formulation of azilsartan medoxomil to improve its aqueous solubility and intestinal permeability. Based on the solubility profile, ethyl oleate, tween 80, and Transcutol P were selected as the oil phase, surfactant, and co-surfactant, respectively. Central composite design (CCD) suggested an optimized azilsartan medoxomil- nanoemulsion formulation (optimized AZL-NE formulation) with 1.25% oil, 15.73% Smix, and 90 s ultrasonication time; it was found to have the droplet size, percentage transmittance, and % cumulative drug release (%CDR) of 71.5 nm, 93.46 ± 1.13%, and 90.14 ± 0.94%, respectively. Furthermore, it exhibited a 0.141 polydispersity index, 34.05 mV zeta potential, a 1.413 ± 0.03 refractive index, 6.68 ± 0.22 pH, 28.17 ± 0.52 cps viscosity, and a 96.98 ± 0.94% percentage drug content. Transmission electron microscopy (TEM) assessed the nano-sized spherical shape, and a differential scanning calorimeter (DSC) assessed the solubilization of the drug in the optimized formulation. The %CDR was 1.71 times higher and the % cumulative drug permeation was 2.1 times higher for the optimized AZL-NE formulation than for the drug suspension through an intestinal segment of a rat, which was also supported by confocal laser scanning microscopy (CLSM) studies. Thus, the nanoemulsion formulation of azilsartan medoxomil ensured the enhancement of the drug availability in the body.

Keywords: azilsartan medoxomil (/search?q=azilsartan+medoxomil); **nanoemulsion (/search?q=nanoemulsion)**; **aqueous solubility (/search?q=aqueous+solubility)**; **bioavailability (/search?q=bioavailability)**; **central composite design (/search?q=central+composite+design)**; **optimization (/search?q=optimization)**

1. Introduction

Azilsartan medoxomil (AZL) is one of the highly selective angiotensin II AT_1 receptor antagonists; it exhibits antihypertensive activity due to the blocking of the direct vasoconstriction caused by the angiotensin II enzyme [**1**]. It has a molecular weight of 568.54 g/mol along with the ionization constant (pKa) and partition coefficient (log P) values of 6.1 and 4.9, respectively [**2**]. It falls under the BCS class II category as it has the aqueous solubility of 0.00978 mg/mL at 37 °C, which also leads to its poor dissolution properties and low bioavailability of 60% [**3**]. Poor aqueous solubility and low bioavailability present the main hindrances to the therapeutic efficiency of azilsartan medoxomil in the treatment of hypertension. It is a prodrug that is hydrolyzed to the azilsartan in the gastrointestinal tract during the absorption phase, and its absorption is not affected by the

presence of food. The maximum drug plasma concentration was achieved within 1.5 to 3 h. It is 99% protein-bound and has a half-life of 11 h [**4**]. Despite poor aqueous solubility and low bioavailability, limited drug delivery systems have been prepared to improve the aqueous solubility and bioavailability of azilsartan medoxomil, including solid dispersions [**5**], nanocrystals [**3**], hydrotrophy, and nanosuspension [**2**]. It shows that various novel drug delivery systems, such as nanoemulsion, solid lipid nanocarriers, nanostructured lipid carriers, dendrimers, and polymeric micelles, are unexplored for their potential to improve the aqueous solubility and bioavailability of azilsartan medoxomil.

Nanoemulsion was selected as a drug delivery system due to its possession of various advantages, such as improved aqueous solubility, enhanced permeability, improved stability, reduced adverse effects leading to higher efficacy, encapsulation of both hydrophilic and hydrophobic drugs, ease of manufacturing, and relatively economical and improved bioavailability [**6**]. The methods used to prepare the nanoemulsion have been categorized into two categories, namely high-energy methods and low-energy methods. The high-energy methods include microfluidization, ultrasonication, and homogenization, whilst the low-energy methods include spontaneous emulsification, phase inversion temperature, and phase inversion composition [**7**]. Various factors, including oil concentration, S_{mix}, and timing of the process, are crucial parameters in the preparation of nanoemulsion, which must be optimized to provide the formulation with better results [**8**]. The optimization of these parameters can be obtained using various optimization techniques, such as the central composite design (CCD), the Box–Behnken design (BBD), the full-factorial design, and the Doehlert matrix (DM), but in pharmaceutical research, CCD and BBD are mainly employed to optimize various independent variables. Moreover, CCD has an advantage over BBD due to the possession of an extra edge, which provides much better predictions. BBD suggests that the formulations have a low, medium, and high value of variables, whilst CCD provides two more extreme values, namely +α and −α which give a rotability to the design [**9**].

2. Results

2.1. Selection of Formulation Components Using Solubility Determination

The solubility profile of AZL in various oils, surfactants, and co-surfactants is given in **Figure 1**. The solubility of AZL in various oils was in the order of ethyl oleate (49.32 mg/mL), Nigella sativa oil (32.33 mg/mL), cod liver oil (28.81 mg/mL), castor oil (16.55 mg/mL), oleic acid (16.45 mg/mL), corn oil (15.18 mg/mL), sunflower oil (12.55 mg/mL), rose oil (9.85 mg/mL), olive oil (9.41 mg/mL), coconut oil (6.66 mg/mL), and semsam oil (4.37 mg/mL) (**Figure 1**A). The oil is a requisite component in the formulation of nanoemulsion to dissolve the hydrophobic drug. However, the concentration of the oil must be as low as possible

in the nanoemulsion formulation because the increase in the concentration of oil leads to an increase in the droplet size, which causes hindrances in the nanoemulsion formulation and a reduction in the physical stability of the prepared nanoemulsion [**10**]. Hence, the oil with the maximum solubility for a drug must be selected. The highest solubility of azilsartan medoxomil in ethyl oleate might be due to the presence of long-chain fatty acids in the structure of ethyl oleate. Moreover, ethyl oleate possesses improved solubilization, a higher nanoemulsion area, and efficacious lymphatic absorption [**11**]. Depending upon the solubility profile, ethyl oleate, Nigella sativa oil, and cod liver oil were selected as the oil phases for the miscibility studies.

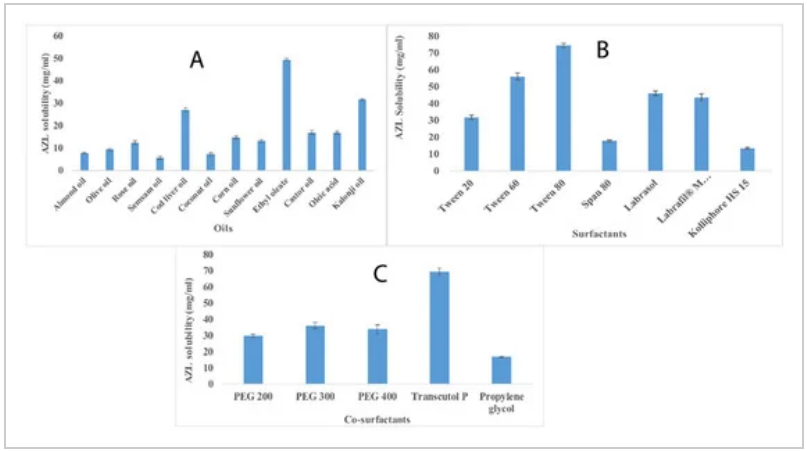


Figure 1. (**A**–**C**) depict the solubility of azilsartan medoxomil in various oils, surfactants, and co-surfactants.

A second important component in the formulation of nanoemulsion is a surfactant that improves the solubility of a drug by minimizing the interfacial tension between the oil phase and the water phase [**12**]. The surfactant was selected based on the solubility and miscibility studies. The solubility of azilsartan medoxomil in various non-ionic surfactants is depicted in **Figure 1**B. The order of solubility of AZL in various surfactants was tween 80 (72.27 mg/mL), tween 60 (52.5 mg/mL), labrasol (45.42 mg/mL), Labrafil® M 1944 CS (43.67 mg/mL), and tween 20 (32.76 mg/mL) (**Figure 1**B). The highest solubility of AZL in tween 80 might be due to its greater capability to reduce the droplet size than the other surfactants owing to its low molecular weight [**13**]. Depending upon the solubility studies, tween 80, tween 60, and labrasol were selected as the surfactants for the miscibility determination.

Another important component in the formulation of the nanoemulsion is the co-surfactant, which is mainly added to improve the emulsification property provided by the surfactant or to reduce the concentration of the surfactant in the formulation

[14]. The order of solubility of AZL in the various co-surfactants was Transcutol P (65.71 mg/mL), PEG 300 (32.28 mg/mL), PEG 400 (28.76 mg/mL), PEG 200 (28.23 mg/mL), and propylene glycol (17.55 mg/mL) (**Figure 1**C). Apart from the highest solubility of AZL in Transcutol P, it is also a good penetration enhancer [**15**]. Transcutol P, PEG 300, and PEG 400 were selected as co-surfactants for the miscibility determination based on the solubility profile.

2.2. Miscibility Determination of Oil, Surfactant and Co-Surfactant

The results of the miscibility determination are summarized in **Table 1**, which depicts that the mixtures having tween 80 as a surfactant and Transcutol P as a co-surfactant were miscible with all the selected oils except cod liver oil (**Figure 2**). Ethyl oleate, tween 80, and Transcutol P were selected as the oil phase, surfactant, and co-surfactant, respectively, to formulate the nanoemulsion because all three of these showed greater drug solubility while being miscible.

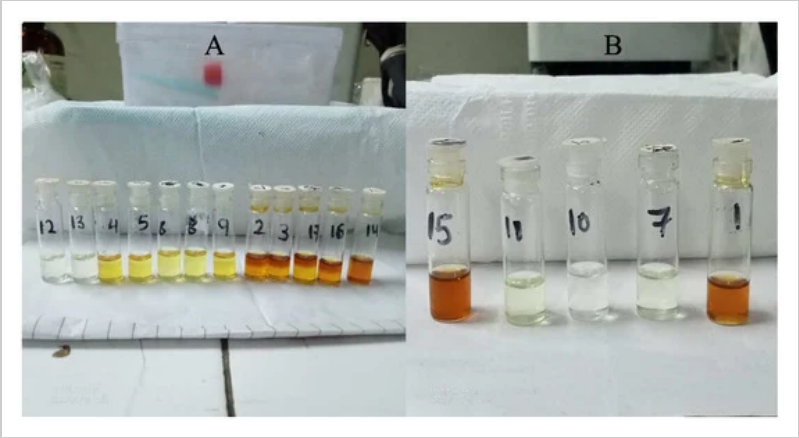


Figure 2. (**A**,**B**) depict the mixtures with phase separation and the clear mixtures, respectively.

Table 1. Results of miscibility determination of selected oils with selected surfactants and co-surfactants.

2.3. Pseudo-Ternary Phase Diagram Studies

The pseudo-ternary diagrams for every S_{mix} ratio are illustrated in **Figure 3**, which depicts the increase in the concentration of the co-surfactant in relation to the surfactant (1:1, 1:2, 1:3, 1:4); the region of the nanoemulsion formulation (clear region) was reduced, whilst there was an increase in the concentration of the surfactant in relation to the co-surfactant (2:1, 3:1, 4:1); the clear region was increased up to the S_{mix} ratio of 3:1, but after this, the increase in the concentration of the surfactant caused a reduction in the clear region (4:1). The enhancement of the clear region with the increase in the concentration of the surfactant might be due to the improved emulsification capacity with the increase in the concentration of surfactant (Tween 80) [**16**]. Moreover, the viscosity of the formulation also increased with an increase in the amount of tween 80, which could be the reason for the reduction in the region of nanoemulsion with the further increase in the concentration of tween 80, as shown by the diagram of S_{mix} 4:1, because the enhanced viscosity of the system harms the droplet distraction and break-up [**13**]. Based on these studies, the S_{mix} in the ratio of 3:1 and the oil: S_{mix} in the ratio of 1:7 showed the maximum region for the nanoemulsion formulation; hence, they were selected for further studies.

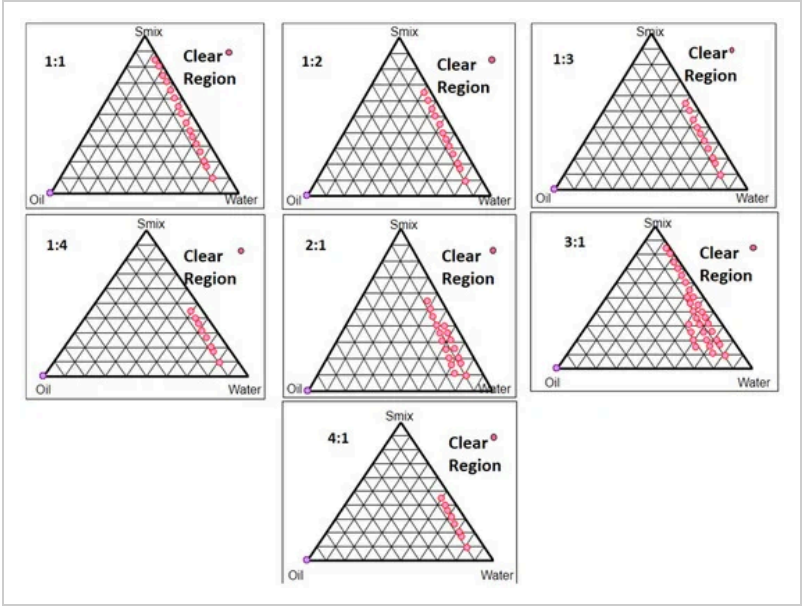


Figure 3. Pseudo-ternary phase diagrams depicting the existence of o/w nanoemulsion region (clear region) for different surfactants: co-surfactant ratios (or S_{mix}).

2.4. Optimization of Formulation Components for Nanoemulsion

A total of 17 different formulations suggested by CCD were prepared using the ultrasonication method, and the values for the droplet size (Y1), %transmittance (Y2), and %CDR (Y3) were in the range of 80.69–311.9 nm, 72.88–93.89%, and 22.06–94.92%, respectively. The experimental values and the predicted values for the dependent variables are summarized in **Table 2**. The optimization software proposed a polynomial quadratic (p = 0.0004 for Y1 and $p < 0.0001$ for Y2 and Y3) for all three of the dependent variables when the observed data for the dependent variables were submitted to it. The predicted R^2 values for all three dependent variables were in agreement with the adjusted R^2 values due to a difference of less than 0.2; these are summarized in **Table 3** along with the mean, standard deviation, and coefficient of variation (%) for all the dependent variables. To predict the quantitative effects of all the independent variables with varying levels on the dependent variables, the software generated the polynomial equations for each response, which are the following:

$$\text{Y1 (droplet size)} = +96.63 + 42.31 \times A - 44.24 \times B - 22.97 \times C - 11.14 \times AB - 6.51 \times AC + 21.05 \times BC + 21.03 \times A^2 + 30.72 \times B^2 + 7.35 \times C^2 \quad (1)$$

$$\text{Y2 (\%transmittance)} = +91.88 - 3.58 \times A + 5.08 \times B + 1.11 \times C + 1.81 \times AB + 0.3000 \times AC - 0.4350 \times BC - 0.8776 \times A^2 - 3.36 \times B^2 - 0.7167 \times C^2 \quad (2)$$

$$\text{Y3 (\% CDR)} = +58.48 - 21.16 \times A + 9.69 \times B + 3.88 \times C - 3.73 \times AB - 4.63 \times AC - 1.39 \times BC - 0.09 \times A^2 - 7.03 \times B^2 - 0.7288 \times C^2 \quad (3)$$

Table 2. CCD-observed AZL-NE formulation experimental runs along with experimental values and predicted values for dependent variables.

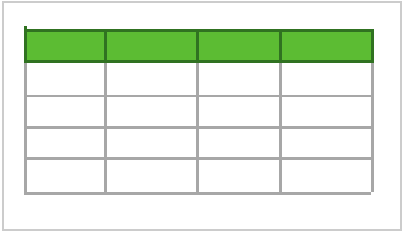


Table 3. Summarized regression analysis data for selected dependent variables.

Equation (1) showed the impact of the oil concentration (A), S_{mix} concentration (B), and sonication time (C) on the droplet size of the formulation. The oil possessed a positive impact whilst the S_{mix} and sonication time possessed a negative impact on the droplet size, which means that with an increase in the oil concentration in the formulation the droplet size will increase, whilst it will decrease with an increase in the S_{mix} concentration and sonication time. The combination of oil*S_{mix} (AB) and oil*sonication time (AC) possessed a negative effect whilst S_{mix}*sonication time (BC) possessed a positive effect on the droplet size, which was also supported by the 3D surface plots (**Figure 4**(A1–A3)) depicting that the increase in the concentration of the S_{mix} and the sonication time causes a decrease in droplet size whilst the increase in the concentration of oil causes an increase in the droplet size. The ANOVA analysis and the model summary statistics for the experimental results provided a greater R^2 of 0.9853 for quadratic Equation (1).

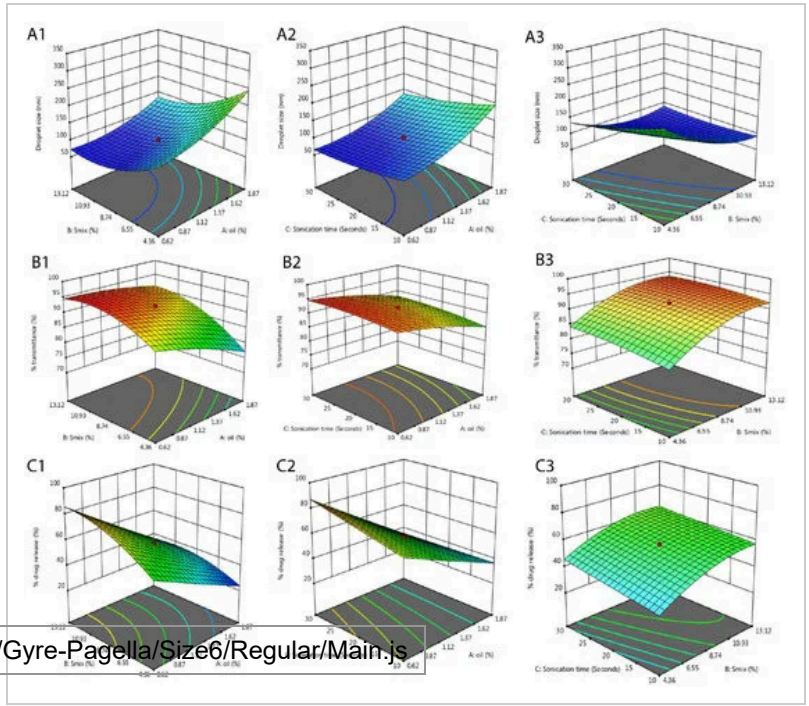


Figure 4. Three-dimensional surface plots depicting the interaction effects of independent variables on droplet size (**A1**–**A3**), % transmittance (**B1**–**B3**), and % CDR (**C1**–**C3**).

By Equation (2) and **Figure 4**B, the % transmittance of the formulation was reduced with an increase in the concentration of oil, but it increased with an increase in the concentration of the S_{mix} and sonication time (**Figure 4**(B1–B3)). The combination of oil*S_{mix} and oil*sonication time possessed a positive impact whilst the S_{mix}*sonication time possessed a negative impact on % transmittance. The ANOVA analysis and model summary statistics for the experimental results exhibited a greater R^2 of 0.9949 for quadratic Equation (2).

Equation (3) and **Figure 4**C displayed the impact of the oil concentration, S_{mix} concentration, and sonication time on %CDR. The %CDR was enhanced with the increase in the concentration of S_{mix} and sonication time, whereas it was reduced with an increase in the concentration of the oil. The combination of the oil*S_{mix}, oil*sonication time, and S_{mix}*sonication time possessed a negative effect on %CDR (**Figure 4**(C1–C3)). These results depict that oil is the main influencing independent variable over S_{mix} and sonication time because a small increase in the percentage of oil causes more of an increase in the droplet size, leading to a hindrance in the drug release. The ANOVA analysis and model summary statistics for the experimental results showed a greater R^2 of 0.9974 for quadratic Equation (3).

The main objective of the CCD-based optimization was to obtain the optimal formulation components to prepare the nanoemulsion. Hence, to obtain it, constraints were applied to the dependent as well as to the independent variables. In-range was selected as the constraint for all the independent variables, whereas the maximum was the constraint for the % transmittance and %CDR and the minimum for the droplet size. Then, the CCD provided the optimized recipe to formulate the formulation and predicted that the formulation must have the oil, S_{mix}, and sonication time of 1.25%, 15.73%, and 90 s, respectively, to obtain the best results for the droplet size, %transmittance, and %CDR of 69.94 nm, 95.10%, and 92.00%, respectively. Then, the formulation suggested by the design was prepared, and it was found that the results of the dependent variables were very close to the results suggested by the design (**Table 4**).

Table 4. Predicted optimized formulation by design along with experimental results.

2.5. Characterization of Optimized Formulation

2.5.1. Droplet Size and PDI Determination

The optimized AZL-NE formulation exhibited an average droplet size of 71.5 nm (**Figure 5**A), which is less than 100 nm, showing that the optimized AZL-NE formulation was better in terms of droplet size and that the smaller droplet size of the nanoemulsion guarantees a better permeation of the drug, resulting in an enhanced surface area. The PDI of the optimized AZL-NE formulation was found to be 0.141 (**Figure 5**A), which showed the narrow size distribution of the droplets in the nanoemulsion formulation. The value of the PDI lies in the range from 0 (perfectly even sample) to 1 (high polydisperse sample), and a PDI value of less than 0.3 is acceptable in the case of nano-formulations [**17**].

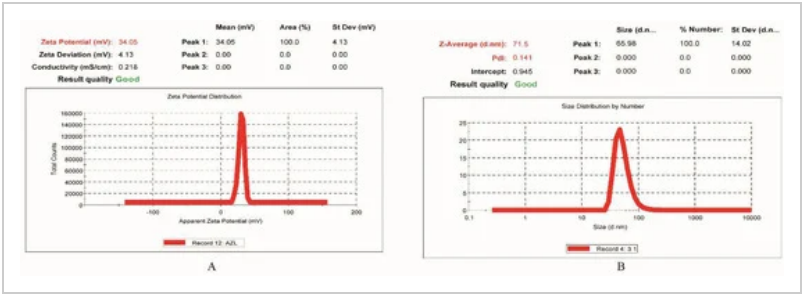


Figure 5. (**A**) depicts the droplet size as well as PDI and (**B**) depicts the zeta potential of the optimized AZL-NE formulation.

2.5.2. Surface Charge (Zeta Potential) Determination

The optimized AZL-NE formulation exhibited a zeta potential of 34.05 mV (**Figure 5**B). The higher value for the zeta potential provides superior repulsion between the droplets in the formulation, leading to a reduction in the chances of the

aggregation of the droplets [**18**]. Moreover, the droplets in a formulation with a zeta potential of higher than +30 mV or −30 mV are considered stable, and hence, the optimized AZL-NE formulation can be considered a stable formulation [**19**].

2.5.3. pH, Viscosity, and Refractive Index Determination

The optimized AZL-NE formulation exhibited a pH of 6.68 ± 0.22, which lies in the pH range for human mucosal tissues (5–6.5), supporting the non-irritant nature of the formulation. The isotropic nature of the formulation was validated by the refractive index, which was found to be 1.413 ± 0.03 for the optimized AZL-NE formulation. The viscosity of the optimized AZL-NE formulation was found to be 28.17 ± 0.52 cps, which is very low. Viscosity is a crucial parameter for deciding on the stability and efficacious drug release from the nanoemulsion [**20**].

2.5.4. Percentage Transmittance Determination

%transmittance was investigated to assess the droplet size as well as the stability of the formulation because any change in %transmittance leads to a change in droplet size as well as the size distribution of the formulation [**21**]. The optimized AZL-NE formulation was found to be 93.46 ± 1.13%, which is close to 100, implying that the formulation was transparent, clear, and able to transmit the light.

2.5.5. Drug Content Determination

The optimized AZL-NE formulation exhibited a drug content of 96.98 ± 0.94%, depicting a higher drug loading capability of the formulation, which is an essential characteristic for nanoemulsion.

2.5.6. Surface Morphology Study Using Transmission Electron Microscopy (TEM)

The results of the surface morphology study using TEM are depicted in **Figure 6**, which reveals that the droplets of the optimized AZL-NE formulation possess a spherical shape, with a size of less than 100 nm. Moreover, no aggregation was present between the droplets of the formulation, which reflects the stability of the formulation. The results of the TEM analysis were in agreement with the droplet size of the formulation determined by the zeta sizer.

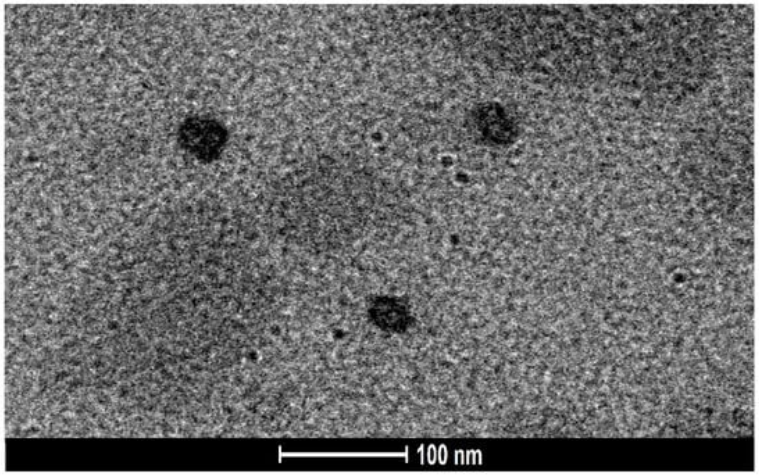


Figure 6. Surface morphology (TEM) image of optimized AZL-NE formulation.

2.5.7. Endorsement of Molecular Dispersion of Drug in Nanoemulsion Using Differential Scanning Calorimetry (DSC)

Figure 7A depicts the DSC peaks for AZL and the optimized AZL-NE formulation. The DSC peak for AZL was obtained at 213.390 °C, which lies within the reference values of 212–214 °C, signifying its purity and crystalline nature. However, the peak of the optimized AZL-NE formulation was obtained at 170.914 °C, which is the peak of the mannitol used as a cryoprotective agent in the lyophilization process of the optimized AZL-NE formulation. The peak of AZL disappeared in the optimized AZL-NE formulation. This confirmed that AZL was completely solubilized in the nanoemulsion and that the crystallization of AZL did not occur during the preparation of the nanoemulsion, signifying the amorphous nature of AZL in the formulation. Apart from this, the DSC peaks demonstrated that AZL and the excipients in the optimized AZL-NE formulation did not have any chemical interaction.

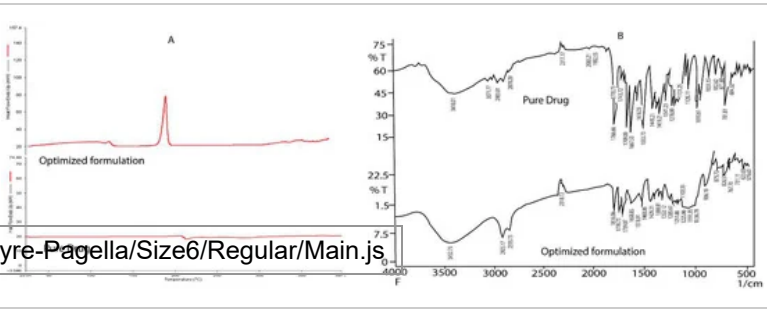


Figure 7. (**A**,**B**) depict DSC thermograms and FTIR spectrum for pure drug and optimized AZL-NE formulation, respectively.

2.5.8. Drug–Excipient Interactions Assessment Employing Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was performed to analyze the physiochemical interactions between the different ingredients of the formulation and the drug. The results of the FTIR spectrum of the pure drug and optimized AZL-NE formulation are depicted in **Figure 7**B. The main peaks of the FTIR spectrum of the pure azilsartan medoxomil were at 3448.01 cm^{-1} (N-H stretching); 3071.77 cm^{-1} (C-H stretching); 1823.77 cm^{-1} (C=O stretching); 1709.00 cm^{-1} (C=O stretching); 1667.53 cm^{-1} (C=O stretching); 1553.73 cm^{-1} (C=C stretching); and 1251.86 cm^{-1} (C-O stretching). The FTIR spectrum of the optimized AZL-NE formulation showed peaks at 3452.73 cm^{-1} (N-H stretching); 2925.17 cm^{-1} (C-H stretching); 1816.06 cm^{-1} (C=O stretching); 1739.87 cm^{-1} (C=O stretching); 1658.85 cm^{-1} (C=O stretching); 1598.91 cm^{-1} (C=C stretching); and 1278.80 cm^{-1} (C-O stretching). There was no major deviation in peaks of the FTIR spectrum for the optimized AZL-NE formulation and pure azilsartan medoxomil drug, which confirmed the absence of molecular interaction between the drug and the formulation components. All the peaks in the FTIR spectrum of the pure drug and the optimized AZL-NE formulation were present as per the functional groups present in the structure of the drug.

2.5.9. In Vitro Drug Release Studies Employing Dialysis Membrane

In vitro drug release studies were carried out to compare the release of AZL from the optimized AZL-NE formulation to the respective suspension (**Figure 8**A). The optimized AZL-NE formulation exhibited a drug release of 53.41 ± 1.26% in the first 30 m, whereas it was only 19.40 ± 0.64% for the drug suspension at the same time. The optimized AZL-NE formulation provided a maximum drug release of 90.14 ± 0.94% in 4 h, followed by deceleration [**22**], whilst the drug suspension provided a maximum drug release of 52.65 ± 0.35% in 8 h. This 1.71-fold increase in the release of AZL from the optimized AZL-NE formulation compared to its suspension could be attributed to the presence of the drug in solution form, owing to the reduced droplet size which will provide an increased surface area for drug dissolution, and an increase in the surface area leads to an increase in the dissolution rate, as suggested by the Noyes–Whitney equation [**23**]. The drug release of the drug suspension being poorer than the optimized nanoemulsion formulation is attributed to the low aqueous solubility of the drug, the coarse particle size, and the presence of the drug in crystalline form. As a result, the optimized AZL-NE formulation boosted the solubility of the drug as well as the dissolution rate due to the reduced droplet size of the nanoemulsion formulation.

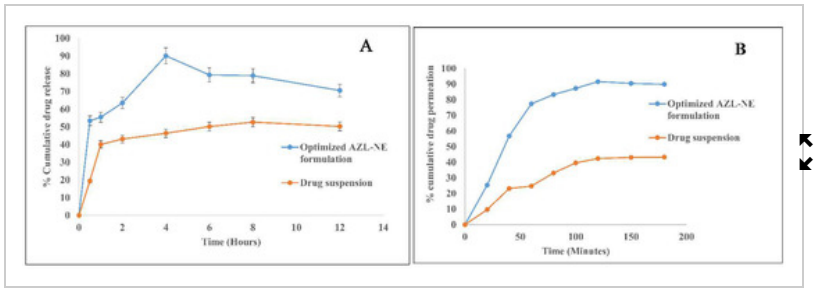


Figure 8. In vitro drug release comparison of optimized AZL-NE formulation with drug suspension (**A**) and ex vivo intestinal permeation comparison of optimized AZL-NE formulation with a drug suspension (**B**).

2.5.10. Ex Vivo Intestinal Permeation Studies

Intestinal permeation studies were evaluated to know the permeation and transit of AZL from the optimized AZL-NE formulation, which was contrasted with the AZL suspension (**Figure 8**B). It was found that the optimized AZL-NE formulation provided a burst of permeation of 77.47% in one hour, whereas the suspension provided only 24.76% at the same time. The optimized AZL-NE formulation exhibited a maximum %cumulative drug permeation of 91.65% in 2 h, whilst the drug suspension exhibited a maximum %cumulative drug permeation of 43.32% in 3 h. The apparent permeability (Papp) for the optimized AZL-NE formulation was 2.32×10^{-4} cm/s, which was significantly greater than the Papp of the drug suspension of 1.07×10^{-4} cm/s. A 2.16-fold increase in apparent permeability through the intestinal membrane could be attributed to an increase in the aqueous solubility of AZL, owing to the nano-size of the optimized AZL-NE formulation; an increase in the aqueous solubility ensures the availability of more drug in a solution form, which will increase the permeation of drug through the intestinal membrane due to its lipoidal nature. Apart from this, the presence of oil improves the permeation of the drug through the lipoidal membrane. The presence of ethyl oleate as a surfactant and Transcutol P is also responsible for the improvement in the permeation of the drugs through the intestinal membrane, owing to their penetration-enhancing properties. Moreover, ethyl oleate is responsible for the improvement in drug absorption due to the inhibition of the P-gp pump present in the enterocytes of the gastrointestinal tract by ethyl oleate [**24**].

2.5.11. Estimation of the Depth of Permeation Using Confocal Laser Scanning Microscopy (CLSM)

CLSM studies were performed to assess the transport of the optimized AZL-NE formulation and the AZL suspension across the enterocytes using rhodamine-B dye. The intensity and depth of rhodamine-B across the enterocytes were

determined by observing the intestinal tissues at the Z axis. The depth of the rhodamine-B fluorescence was observed up to 25 µm in the case of the optimized AZL-NE formulation (**Figure 9**A), whilst it was decreased to 10 µm in the case of the AZL suspension (**Figure 9**B), signifying a 2.5- fold enhancement in the permeation of AZL from the optimized AZL-NE formulation compared to its suspension. This enhancement in permeation is possible due to the nano-size of the droplets in the nanoemulsion formulation. Apart from this, previous studies have reported that surfactants with an HLB value of 10–17 improve the absorption and permeability of drugs by inhibiting the P-gp efflux pump [**25**]. Hence, in this research, the improved permeation of AZL from the optimized AZL-NE formulation might be due to the presence of tween 80 with an HLB value of 15. Moreover, the improved permeation could also be due to the presence of Transcutol P as a co-surfactant.

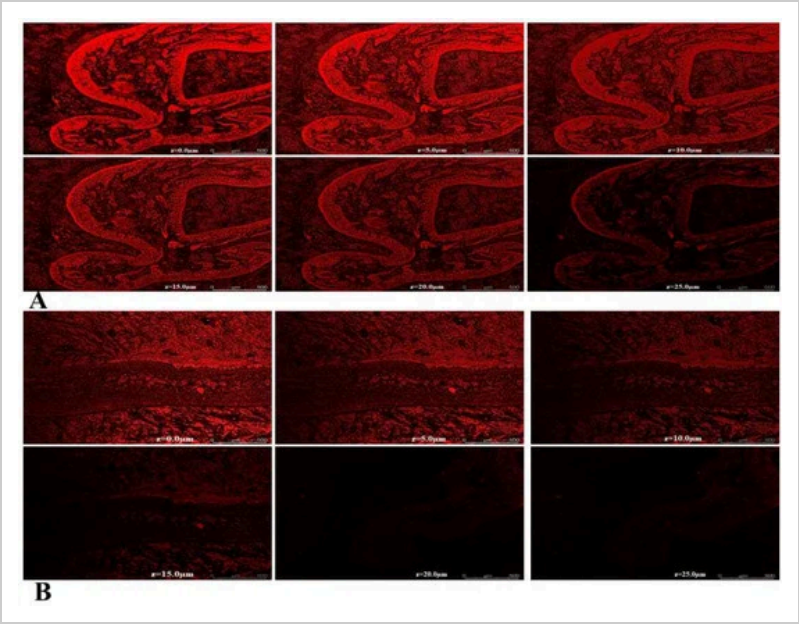


Figure 9. Image of CLSM for intestinal tissues treated with AZL-NE (**A**) and drug suspension (**B**).

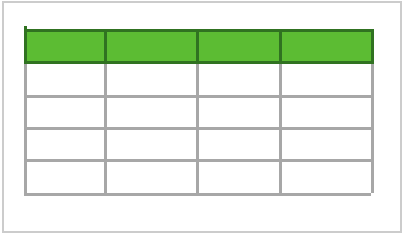
2.5.12. Assessment of Stability using Thermodynamic Stability and Storage Stability

The major issue with nanoemulsion formulation is maintaining its stability throughout the shelf life. Hence, to verify it, stability testing was performed. The results of the thermodynamic stability studies demonstrated that there was no phase separation on the exposure of the optimized AZL-NE formulation to heating–cooling cycles, centrifugation tests, and freeze–

thaw cycles. However, minor creaming in the optimized formulation was observed, which was redistributed after minor shaking of the formulation.

The results of the storage stability studies demonstrated that the optimized AZL-NE formulation has no sign of phase separation on visual inspection when stored in different storage conditions. The change in droplet size, % transmittance, and %CDR was minor for the optimized AZL-NE formulation when stored at 25 ± 1 °C and 4 ± 1 °C. The data generated after the storage stability studies for the optimized AZL-NE formulation are summarized in **Table 5**, depicting a better storage stability for the optimized AZL-NE formulation.

Table 5. Data of stability studies for optimized AZL-NE formulation.



3. Materials and Methods

3.1. Materials

Azilsartan medoxomil was obtained from Alkem laboratories limited, Mumbai, India as a gift sample. Almond oil, olive oil, rose oil, semsam oil, cod liver oil, coconut oil, castor oil, Nigella sativa oil, corn oil, sunflower oil, tween 20, tween 60, tween 80 were purchased from SD Fine chemicals (Mumbai, India). Ethyl oleate, oleic acid, span 80, labrasol, Labrafil® M 1944 CS, kolliphore HS, and Transcutol P were obtained as gift samples from Gattefosse, Mumbai, India. PEG 200, PEG 300, PEG 400, and propylene glycol were procured from Acros organic, Mumbai, India. Potassium dihydrogen phosphate and disodium hydrogen phosphate, rhodamine B, methanol, and mannitol were procured from Sigma-Aldrich (New Delhi, India). All the chemicals used for experimentation were of analytical grade.

3.2. Methods

3.2.1. Selection of Formulation Components Using Solubility Determination

The solubility of azilsartan medoxomil was determined in various formulation components, namely oils, surfactants, and co-surfactants, to select the best materials required for the preparation of nanoemulsion. It was carried out in a variety of oils (almond oil, olive oil, rose oil, semsam oil, cod liver oil, coconut oil, corn oil, sunflower oil, ethyl oleate, castor oil, oleic acid, and Nigella sativa oil), surfactants (tween 20, tween 60, tween 80, span 80, labrasol, Labrafil® M 1944 CS and kolliphore HS 15) and co-surfactants (PEG 200, PEG 300, PEG 400, Transcutol P, and propylene glycol) by dissolving the excess quantity of the drug in 2 mL of various oils, surfactants, and co-surfactants separately in 2.5 mL eppendrof tubes, followed by continuous vortexing for 48 h at room temperature to attain the equilibrium. Then, the samples were centrifuged at 3000 rpm for 15 min, and the supernatant was collected. The drug was extracted from the supernatant using methanol, and analysis was carried out by UV Spectrophotometer (UV 1700, Shimadzu, Japan) [**26**,**27**].

3.2.2. Miscibility Determination of Oil, Surfactant, and Co-Surfactant

The miscibility of the oil phases was determined with surfactants and co-surfactants selected on the basis of the solubility profile by mixing at the ratio of 1:1, followed by vortexing for approximately 15 min. Then, the samples were kept without any disturbance for 24 h and evaluated visually for phase separation or any visible color changes. The mixtures that appeared clear were selected for further studies [**28**].

3.2.3. Pseudo-Ternary Phase Diagram Studies

The main purpose of the pseudo-ternary phase diagram studies is to identify the optimized ratio of the selected surfactant and co-surfactant required for the preparation of nanoemulsion [**29**]. Different ratios (1:1, 1:2, 1:3, 1:4, 2:1, 3:1, and 4:1) of surfactant and co-surfactant (S_{mix}) were mixed with selected oil in different ratios of 9:1, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1, followed by titration of each mixture of oil and Smix against the deionized water under continuous stirring. After each addition of the deionized water to the mixture, visible observation for clarity and turbidity was performed. Those systems that seemed transparent and easily flowable were marked on a pseudo-three-component phase diagram representing the aqueous phase, the second representing the oil, and the third representing the S_{mix} ratio, utilizing the CHEMEX School software ver 3.51 (Arne Standnes, USA) [**30**].

3.2.4. Preparation of Drug-Loaded Nanoemulsion

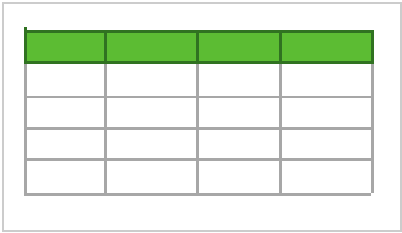
AZL-loaded nanoemulsion was prepared using the ultrasonication method [**13**,**31**]. In this method, the fixed amount of the drug was filled into the glass vial with the required amount of the oil, followed by the dissolution of the drug in the oil along with

the continuous addition of S_{mix}. After that, the obtained mixture was micro-titrated with distilled water to provide an emulsion with coarse droplets. To convert the coarse emulsion into nanoemulsion, ultrasonication was employed for 30–90 s, using the ultrasonic processor of 30 kHz (Hielscher-Ultrasound Technology, Germany) [**21**]. Ultrasonication may cause cavitation, resulting in heat production; hence, the sonication was performed in seconds [**32**].

3.2.5. Optimization of Formulation Components for Nanoemulsion

Central composite design (CCD) was employed to optimize the main components required for the preparation of the nanoemulsions, using Design Expert software, Stat-Ease, Minneapolis, MN, USA [**13**]. The optimization was carried out using oil (%), S_{mix} (%), and sonication time (S) as independent variables, depending upon their potential to influence the dependent variables, namely droplet size (Y1; nm), %transmittance (Y2; %), and %CDR (Y3; %). Amongst the independent variables, oil (%) and Smix (%) were formulation variables and sonication time (s) was the process variable, which varied by three concentrations (high, medium, and low), as suggested by pseudo-ternary phase diagram studies [**33**]. The design suggested 17 formulation runs amongst 3 central points, 8 factorial points, and 6 axial points. The independent variables and dependent variables along with their levels are summarized in **Table 6**.

Table 6. Selected independent variables along with their levels and dependent variables.



3.2.6. Characterization of Optimized Formulation

Droplet Size and Polydispersity Index (PDI) Determination

The droplet size of nanoemulsion is a crucial characteristic as smaller particles will produce more surface area, which will eventually improve the drug's absorption. The droplet size and PDI of the optimized AZL-NE formulation were determined using the dynamic light scattering technique (Zetasizer 1000 HAS, Malvern Instruments, Malvern, UK). The procedure involved the

dilution of the formulation ten times with double distilled water; it was placed in a quartz cuvette for the determination, at an angle of 90° and a temperature maintained at 25 °C [**34**].

Surface Charge (Zeta Potential) Determination

The surface charge or zeta potential of the optimized AZL-NE formulation was determined using the Zetasizer 1000 HAS, Malvern Instruments, UK. The procedure involved the dilution of the formulation ten times with double distilled water; it was placed in a quartz cuvette for the determination of the surface charge [**34**].

pH, Viscosity, and Refractive Index Determination

The pH of the formulation was determined at 25 ± 0.5 °C, using a previously calibrated digital pH meter (Mettler Toledo, Langacher, Switzerland). The viscosity was determined by a Brookfield viscometer (DV-III+ Rheometer, Brookfield, WI, USA), employing a cone and plate at a temperature of 25 °C and speed of 10 rpm [**35**]. The refractive index was determined using an Abbe refractometer(Nirmal International, India) at room temperature [**36**].

Percentage Transmittance Determination

Percentage transmittance indicates the clarity of the formulation. It was determined spectrophotometrically using a UV-VIS spectrophotometer (Shimadzu, Japan). In this, 1 mL of the formulation was diluted 100 times with distilled water, followed by analysis at the wavelength of 247 nm [**37**].

Drug Content Determination

The drug content of the optimized AZL-NE formulation was determined by dissolving 1 mL of formulation in a suitable quantity (10 mL) of methanol. This mixture was then shaken for 30 min at 50 rpm at 37 ± 0.5 °C in an incubator (LSI-2005 RL, Lab Tech Co., Seoul, Korea), followed by the collection of the supernatant liquid after 30 min; it was then analyzed using a UV spectrophotometer (Shimadzu, Japan) at 247 nm and methanol as a blank [**38**].

Surface Morphology Study Using Transmission Electron Microscopy (TEM)

The surface morphological characterization of the optimized AZL-NE formulation was performed using transmission electron microscopy (TEM) (Tecnai G20 HR-TEM. Thermo Scientific, Waltham, MA, USA). It was performed by placing one drop of formulation on wax paper and then transferring it to the copper grid (300 mesh), stained with 2% *w*/*v* phosphotungstic

acid, followed by air drying. Then, the dry sample was displayed under the transmission electron microscope to evaluate the size and shape of the droplets of the formulation [**18**].

Endorsement of Molecular Dispersion of Drug in Nanoemulsion Using Differential Scanning Calorimetry (DSC)

The DSC thermograms of the pure drug and optimized formulation were obtained using DSC Perkin Elmer. To obtain the thermogram of the optimized formulation, the lyophilization of the formulation was performed using mannitol as a cryoprotective agent at a concentration of 5% due to the possession of higher solubility in water and also the conversion into the crystalline form on freeze-drying. To obtain the DSC thermograms, 2 mg of pure drug and lyophilized powder of formulation were sealed in an aluminum pan separately. Then, the DSC was performed over the temperature of 30 to 300 °C, along with a heating rate of 10 °C/min in an air environment. The flow of nitrogen gas was maintained at 60 mL/min [**21**].

Drug–Excipient Interactions Assessment Employing Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analysis of the pure drug and the optimized formulation was carried out employing the potassium bromide pellet technique. In this technique, a homogenous mixture was made by adding samples of pure drug and the lyophilized form of the optimized formulation separately with potassium bromide at a ratio of 1:10, followed by the grinding of the mixture. Then, a small amount of powdered mixture was compressed to a thin, semitransparent film (pellets) by the application of pressure. The pellet's IR spectrum was assessed between 500 and 4000 cm^{-1} using the FTIR spectrophotometer [**21**].

In Vitro Drug Release Studies Employing Dialysis Membrane

Drug release studies were implemented to compare the drug release of the optimized AZL-NE formulation with a drug suspension. In this, one milliliter of optimized AZL-NE formulation and drug suspension was filled into the separate dialysis bag, which was then dipped into the beaker with 80 mL of simulated intestinal fluid of pH 6.8 [**18**]. The beaker was kept in a magnetic stirrer, which was operated at 150 rpm and 37 ± 0.5 °C. At scheduled time intervals (30, 60, 120, 240, 360, 480, and 720 min), an aliquot of 5 mL was collected from the beaker, followed by the addition of fresh dissolution medium to the beaker to maintain the sink conditions. The collected samples were carried out for analysis using a validated UV-spectrophotometer (UV 1700, Shimadzu, Japan) at a wavelength of 247 nm [**39**]. The cumulative drug release from the optimized AZL-NE formulation and drug suspension was determined using the formula:

$$\%\,CDR = \frac{DR}{IDC} \times 100 \quad (4)$$

The obtained values were plotted as %CDR versus time.

Ex Vivo Intestinal Permeation Studies

Ex vivo intestinal permeation studies of the optimized AZL-NE formulation and drug suspension were performed using the non-inverted rat sac model [**40**]. In this study, a female Wistar rat weighing between 100 and 150 g was kept in standard conditions with free access to clean drinking water. The ileum portion of the small intestine was excised and cleaned of facial debris using Tyrode's solution. The cleaned section of the small intestine was converted into the sac, tying one end firmly using thread, followed by insertion of the optimized AZL-NE formulation and drug suspension separately at a defined concentration in the sac and then also tying another end firmly using thread. The sac was then placed in a jacketed glass with 40 mL of Tyrode's buffer (dissolution medium, pH 6.8) which had been previously warmed to 37 ± 0.5 °C and oxygenated for 120 min with 95% oxygen through an aerator. At specified time intervals (20, 40, 60, 80, 100, 120, 150, and 180 min), a sample of 4 mL was collected, followed by the addition of a fresh dissolution medium to maintain the sink conditions. All the collected samples were filtered through the membrane filter with a pore size of 0.45 µm and then analyzed using the UV spectrophotometer. The amount of the drug that escaped within 180 min from the optimized AZL-NE formulation was compared to the drug suspension and plotted as % cumulative drug permeation versus time [**41**]. The apparent permeability (P_{app}) was determined using the formula:

$$P_{app} = \frac{dQ}{dt} / \quad A \times Ci \tag{5}$$

where (dQ/dt), A, and Ci denote the rate of drug permeation outside the sac, the surface area of the sac, and the initial concentration into the sac, respectively.

Estimation of the Depth of Permeation Using Confocal Laser Scanning Microscopy (CLSM)

CLSM is a useful method for increasing the visibility, penetration, and depth of nano-formulation in various tissues. Using optical laser scans produces deeper-selective, higher-resolution images of the tissue or the gut that are superior to fluorescence or optical microscopy. In this study, a female Wistar rat was used and sacrificed using cervical dislocation. Then, 4 cm-long ileum portions were excised from the small intestine followed by cleaning using a normal saline solution to remove all facial debris. The intestinal sacs were filled with a sample of rhodamine B-labeled optimized AZL-NE formulation and rhodamine B-labeled drug suspension using the syringe, followed by the tying of both ends of the intestinal sac firmly using thread. These intestinal sacs were kept in Tyrode's buffer at the temperature, speed, and oxygen supply of 37 ± 0.5 °C, 45 rpm,

and 95%, respectively, for 3 h [**42**]. The intestinal sacs were then cut lengthwise into min species and inserted into slits after being washed with saline solution to remove any remaining free rhodamine dye. The depth of permeation of the rhodamine B across the z-axis of the intestinal wall was estimated using confocal laser scanning microscopy (Leica Microsystems SP8, Mumbai, India) [**18**].

Assessment of Stability Using Thermodynamic Stability and Storage Stability Studies

The stability and phase reliability of the optimized AZL-NE formulation was evaluated using thermodynamic stability studies under varying conditions of temperature and centrifugal force. Under the thermodynamic stability studies, three distinctive tests were performed, namely heating–cooling cycles, centrifugation tests, and freeze–thaw cycles. In the heating and cooling cycles, the formulation was conducted in six cycles at 4 °C and 45 °C for 48 h each, followed by observation for phase separation. After this, a centrifugation test was performed in which the formulation was subjected to centrifugation at 3500 rpm for 30 min, followed by observation for phase separation. Then, freeze–thaw cycles were carried out, in which the formulation was subjected to six cycles of −21 °C and 25 °C for 48 h each and observed for phase separation [**43**].

The storage stability of the optimized AZL-NE formulation was assessed for one month, during which the formulation was packed in glass vials (amber colored) and stored at different conditions of temperature (25 ± 1 °C, room temperature, and 4 ± 1 °C, refrigeration), followed by an examination of the formulation at a specified time interval (10, 20, and 30 days) for phase separation, creaming, droplet size, % transmittance, and %CDR [**39**].

4. Conclusions

AZL-loaded nanoemulsion was prepared and optimized successfully using a central composite design to improve the aqueous solubility and intestinal permeation of the drug. The optimized AZL-NE formulation exhibited the droplet size, % transmittance, and %CDR of 71.5 nm, 93.46 ± 1.13%, and 90.14 ± 0.94%, respectively. The optimized AZL-NE formulation provided a drug release that was 1.71 times higher than the drug suspension. The drug permeation from the optimized AZL-NE formulation was 2.1-fold greater than the drug suspension through the intestinal segment of a rat. The results of CLSM also showed a deeper penetration of the optimized AZL-NE formulation than the drug suspension. All these results showed the superiority of nanoemulsion in the improvement of the aqueous solubility and intestinal permeability of hydrophobic drugs. The observed results opened the door for additional research, in which pharmacokinetics and pharmacodynamics studies will be conducted to approve the bioavailability and in vivo potential of the drug. In conclusion, this study effectively optimized AZL-NE

via a central composite design, which demonstrates the prospective potential for improving intestinal permeability and may help to improve the oral bioavailability of azilsartan medoxomil.

Author Contributions

G.K.: data curation, writing—original draft, methodology, formal analysis; T.V.: supervision, writing—review and editing; K.P.: conceptualization, supervision; O.A.K. and A.S.: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The approval for the protocol of the animal studies was given by the Institutional Animal Ethical Committee of Anand College of Pharmacy, India (approval no. CPCSEA/IAEC/ACP/19).

Informed Consent Statement

Not applicable.

Data Availability Statement

Not applicable.

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Conflicts of Interest

No conflict of interest is declared by the authors.

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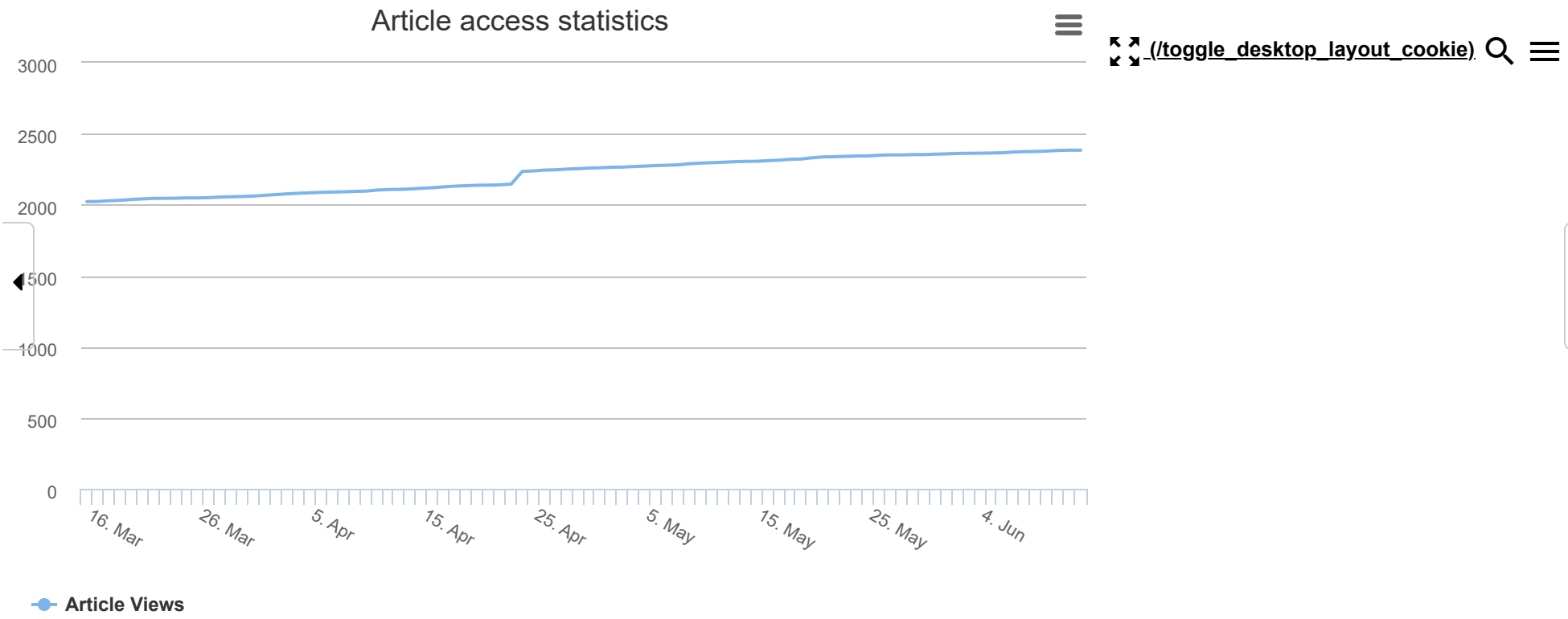

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Insights on Development Aspects of Polymeric Nanocarriers: The Translation from Bench to Clinic

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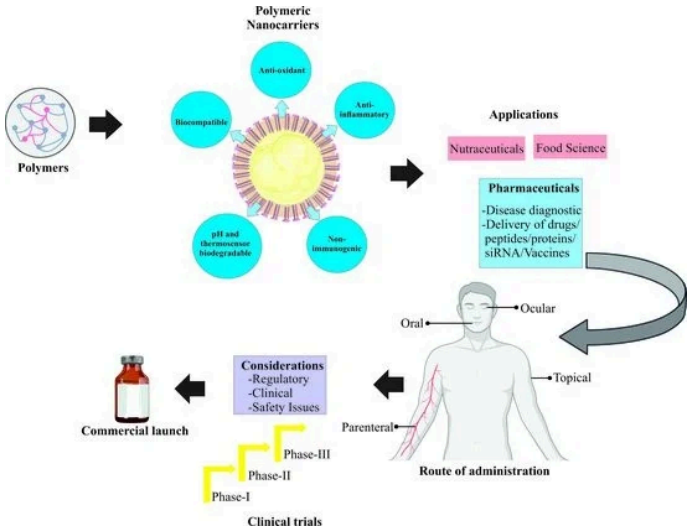
Abstract

Scientists are focusing immense attention on polymeric nanocarriers as a prominent delivery vehicle for several biomedical applications including diagnosis of diseases, delivery of therapeutic agents, peptides, proteins, genes, siRNA, and vaccines due to their exciting physicochemical characteristics which circumvent degradation of unstable drugs, reduce toxic side effects through controlled release, and improve bioavailability. Polymers-based nanocarriers offer numerous benefits for in vivo drug delivery such as biocompatibility, biodegradability, non-immunogenicity, active drug targeting via surface modification, and controlled release due to their pH—and thermosensitive characteristics. Despite their potential for medicinal use, regulatory approval has been achieved for just a few. In this review, we discuss the historical development of polymers starting from their initial design to their evolution as nanocarriers for therapeutic delivery of drugs, peptides, and genes. The review article also expresses the applications of polymeric nanocarriers in the pharmaceutical and medical industry with a special emphasis on oral, ocular, parenteral, and topical application of drugs, peptides, and genes over the last two decades. The review further examines the practical, regulatory, and clinical considerations of the polymeric nanocarriers, their safety issues, and directinos for future research.

Keywords: polymeric nanocarriers (/search?q=polymeric+nanocarriers); **polymersomes (/search?q=polymersomes)**; **nanosized hydrogels (/search?q=nanosized+hydrogels)**; **cubosomes (/search?q=cubosomes)**; **ocular (/search?q=ocular)**; **parenteral (/search?q=parenteral)**



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Graphical Abstract

1. Introduction

A polymer represents several repeating units leading to the formation of a compound that has characteristics of high relative molecular mass and associated properties. In 1833, Jons Jakob Berzelius coined the term “polymer” which originated from the Greek word (polus, meaning “many, much”) and (meros, meaning “part”). Following major developments in polymer fabrication, the molecular properties of polymers were only recognized after the efforts of Hermann Staudinger in 1922, who first suggested that polymers consist of long chains of atoms bound together by covalent bonds. This took more than a decade to achieve acceptance among the scientific community. Initially, polymers were investigated according to the theory of associations or composite theory that emerged in 1861. It was suggested that cellulose and other polymers were colloids and molecular aggregates with small molecular weights bound by an unidentified intermolecular force. The use of polymers is not new in the medical field [**1**].

Natural polymers are being used for decades as ingredients for herbal remedies. The case is very different when it comes to synthetic polymers. Water-soluble polymers can be considered a modern accomplishment as macromolecular drugs or as part of the inoculation-related drug delivery systems. The first polymer-drug conjugate in form of mescaline-N-vinylpyrrolidone conjugates was synthesized in 1955. A decade later, it was predicted that polyethylene glycol (PEG) can be attached to proteins using a method called PEGylation that comprises adding the covalent bond of polymer (ethylene glycol) polymer chains to some other entity, normally a therapeutically active drug or protein. In 1994, the first synthetic polymer-drug conjugate was clinically proven to treat cancer. This was made up of a doxorubicin copolymer conjugation of (N-(2-hydroxypropyl) methacrylamide [**2**]. In 1977, Davis and Abuchowski described first-time PEGylation for drug delivery via the covalent attachment of PEG to bovine serum albumin and liver catalase proteins [**3**]. They reported that it could increase the systemic circulation time and decrease the immunogenicity of the proteins without significantly compromising activity. In 1990, the FDA approved the first PEGylated protein product, Adagen®, a PEGylated adenosine deaminase enzyme for severe combined immunodeficiency disease. Doxil® is the first FDA-approved PEGylated nanoparticle (NP) based product that was commercialized in 1995. At present, eight other PEGylated protein therapeutics have been FDA approved for various diseases [**4**]. The success of protein PEGylation as a method for producing longer circulating, and thus more efficacious intravenous therapies, led to investigations of nanoparticle (NP) PEGylation for systemic applications in the early 1980s and 1990s [**5**]. Recognized as foreign objects, they are readily excreted from the systemic circulation by the cells of the mononuclear phagocyte system, precluding accumulation in target cells and tissues [**6**,**7**,**8**]. Block copolymer-based micelles have also been explored for targeting specific cancer cells as these micelles are capable of trapping the drug or connecting it covalently to the co-polymers. Two polymer-protein complexes, PEG-interferon-α (an antiviral medication meant to treat liver cirrhosis) and PEG granulocyte colony-stimulating factor were put on the market around the 2000s [**9**]. The first therapeutic nanoparticles (albumin-trapped paclitaxel) were licensed for metastatic breast cancer treatment after five years [**10**]. Since then, polymeric micelles and nanoparticles comprising block copolymers have emerged as a storm and impacted clinical care. Some of the polymeric micelles have been approved and are currently being marketed in Korea and Europe. Genexol-PM is a polymeric micellar formulation of paclitaxel got approved in 2007 and is marketed under the name Cynviloq by Sorrento Pharmaceutics [**11**]. In 2013, NanoCarrier Co. Ltd. Tokyocompleted Phase II clinical studies of Nanoplatin (NC-6004) which is a polymeric micellar formulation of cisplatin [**12**]. NK012 of Nippon Kayaku Co. Ltd. Tokyo, is another polymeric micelle formulation of SN-38 (an irinotecan metabolite) which has completed Phase II clinical trials in 2015 and received orphan drug designation from USFDA [**13**]. In 2017, Nippon Kayaku Co., Ltd., Tokyo completed another clinical Phase III study for NK105 which is a polymeric

micellar formulation of paclitaxel consisting of PEG and modified polyaspartate as a hydrophobic block. Currently, Docetaxel-loaded polymeric micelles plus Oxaliplatin is under Phase II clinical trial for patients with esophageal squamous cell carcinoma. Epirubicin-loaded polymeric nanoparticles is undergoing a Phase I/II clinical trial for patients having tumors or soft tissue sarcoma. Another polymeric nanoparticle loaded with Cetuximab is undergoing a Phase I clinical trial for colon cancer [**14**]. All of the above accomplishments represent the central factor that led to the growth of polymer-based pharmaceutical products, viz. nanoparticles, nanoconjugates, and micelles.

Nanomaterials took the pharmaceutical, nutritional supplements, and food industries by storm. Such structures are 1–100 nm (10^{-9} m) in diameter and have distinct physicochemical properties in comparison to macromaterials [**15**]. Nanotechnology is one of the most innovative technologies and is capable of improving food safety and quality. However, there are many studies showings that nanomaterials are not inherently harmless [**16**]. Nanomaterials are used for therapeutics in the healthcare sector, treatment for cancer, and diagnosis of disease. But in addition to their advantages, they also possess several undesirable effects, among which an increase in the absorption rate is the most important [**17**,**18**,**19**]. They increase the absorption rate as a result of the increased surface area of the nanomaterials, which allows increased absorption rate by the surrounding tissues leading to the generation of several unintended results [**20**]. The increased absorption rate is often seen in powder nanomaterials but less so in solids. However, nanomaterials are used to minimize these effects through controlled absorption [**21**]. Polymers possess unique qualities, including biocompatibility and biomimetic properties, which is why they are widely applicable in biomaterials as biosensors, catalysis, and the delivery of medicines [**22**]. Their use in medicines is beneficial due to their ability to distribute the therapeutic compound efficiently to the active site or target, making drug safety and efficacy certain [**23**].

2. Types of Polymer Based Nanocarriers

The precise classification of the nanocarriers is a complicated task to attempt. In the arena of biomedical and pharmaceutical nanotechnology, no specific boundaries have been defined, and therefore different perceptions may be relevant. However, a concise sketch of potential polymer-based nanocarriers is important as depicted in **Figure 1**. Depending on their origin they can also be identified as natural and synthetic polymeric nanoparticles. These are distinguished by their concurrent polymeric design and colloidal (1–1000 nm) measurements and have been commonly identified as polymeric

(biodegradable and biocompatible) nanoparticles, dendrimers, nanoemulsions, polymersomes, polymeric micelles, or biopolymer complexes or cubosomes [**24**].

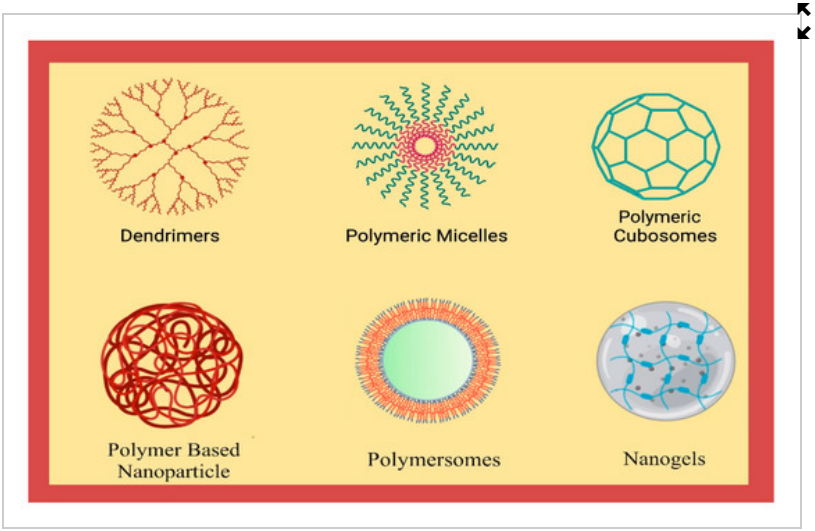


Figure 1. Diagrammatic representation of different polymeric nanocarriers.

The highly relevant articles were retrieved via various search engines on the databases, Science Direct, Web of Sciences, PubMed, Scopus, PubChem, and Google Scholar. The examples have been selected on the basis of their significance to provide experimental insight into each type of nanocarriers to the readers. The keywords and phrases used for the search are “dendrimers”, “polymeric micelles”, “polymerosomes”, “nanogels”, “dendrimers”, “cubesomes”, “NLCs”, “nanographene”, “nanocomposites”, “CNTs”, “polymers”, and “nanocarriers”, “In vitro and in vivo activities”. The inclusion criteria implied for selecting particular polymer-based nanocarriers represent the significance of that particular study.

2.1. Polymer-Based Nanoparticles

Nanoparticles are sub-nanosized colloidal structures composed of synthetic or semisynthetic polymers. NPs range in size between 1 and 100 nm [**25**]. The ultra-small size of NPs exhibits a larger surface area to volume ratio and hence allows greater drug loading within the core via encapsulation and onto the surface via absorption. Their unique size range allows for extended circulation, leading to prolonged drug release. The entrapment of drugs in NPs protects against chemical degradation and improves their pharmacokinetic properties, leading to controlled release over a long period. NPs exhibits flexibility of surface

functionalization with targeting ligands for site-specific drug delivery and thereby improve therapeutic efficacy [**26**]. Materials used in the manufacturing of nanocarriers based on polymers are selected not only for their functional characteristics, such as their capability to structure planned nanostructures, but also for their biological behavior. Polymers that are biodegradable and biocompatible are usually favored. Given the multitude of accessible or easily synthesizable polymers with promising potential for medicinal use, regulatory approval has been achieved for just a few. Various categories and forms of polymers including poly-lactic-co-glycolic acid (PLGA), poly-DL-lactic acid (PDLLA), polycaprolactone (PCL), polyacrylate, polymethacrylate, cellulose by-products, poly(ethylene oxide) triblock (PEO)/polypropylene oxide (PPO) (PEO-PPO-PEO; poloxamers), PEG, polyvinyl alcohol (PVA) and alginate was licensed by the United States Food and Drug Administration (USFDA) and other foreign authorities for various medical purposes over the years [**27**,**28**].

However, not all of these have sufficient features to develop nanomaterials that can act as nanocarriers, so unsurprisingly previously authorized materials may be standard candidates for rapid clearance by regulatory agencies. In comparison, modern polymer conjugates and polymers need rigorous biological and toxicological assessment to be approved for human use, and their clearance may be overwhelming [**29**].

Yaşar et al., 2020 developed polymer-based NPs with 74 nm particle size for selective protein recognition by using thiol-ene miniemulsion photopolymerization. The developed NPs revealed more selectivity toward myoglobin [**30**].

Puri et al., 2017 fabricated pH-sensitive polymer-based NPs to deliver bioactive compounds employing a dispersion polymerization approach. They reported that the rate of hydrolysis and drug discharge were quicker at pH 5.0 compared to pH 7.4 [**31**].

2.2. Polymeric Micelles

Fabricated by amphiphilic block copolymers, the polymeric micelles can be self-assembled (and disassembled) under a valid concentration or temperature environment as versatile spherical structures (i.e., nanosized core-shell molecules produced by self-association of amphiphilic block copolymers when exposed to an aqueous solvent). In nature, the core of micelles is hydrophobic and appropriate for incorporating hydrophobic moieties [**32**]. They are suitable for parenteral delivery compared to current solubilizing agents (e.g., cremophor EL), causing the dosage of effective but toxic and poorly water-soluble compounds to increase [**33**]. Investigations into nano-bio dynamics which can function at the molecular, cellular, and tissue levels is often ignored [**34**].

Varshosaz et al., synthesized docetaxel-loaded polymeric micelles using folic acid grafted synperonic PE/F 127-cholesteryl hemisuccinate copolymer for precise delivery to cancerous cells. The in vitro cellular uptake observations in B16F10 melanoma cells revealed improved uptake of targeted polymeric micelles through folic acid receptor-mediated endocytosis [**35**]. The micelles were found to be non-toxic and safe for biomedical usage over a range of 0.001 to 500 µg/mL. The morphological examination revealed the smooth spherical shape of the micelles. In vitro drug release investigation showed an initial burst trailed by slower and sustained release up to 134 h. The early burst release could be credited to the quick dissolution of surface-bound drugs followed by steady diffusion from the core of micelles. An in vivo antitumor efficiency study in B16F10 melanoma-bearing mice revealed effectively inhibited tumor growth by the drug-loaded targeted polymeric micelles compared to non-targeted polymeric micelles as shown in **Figure 2**.

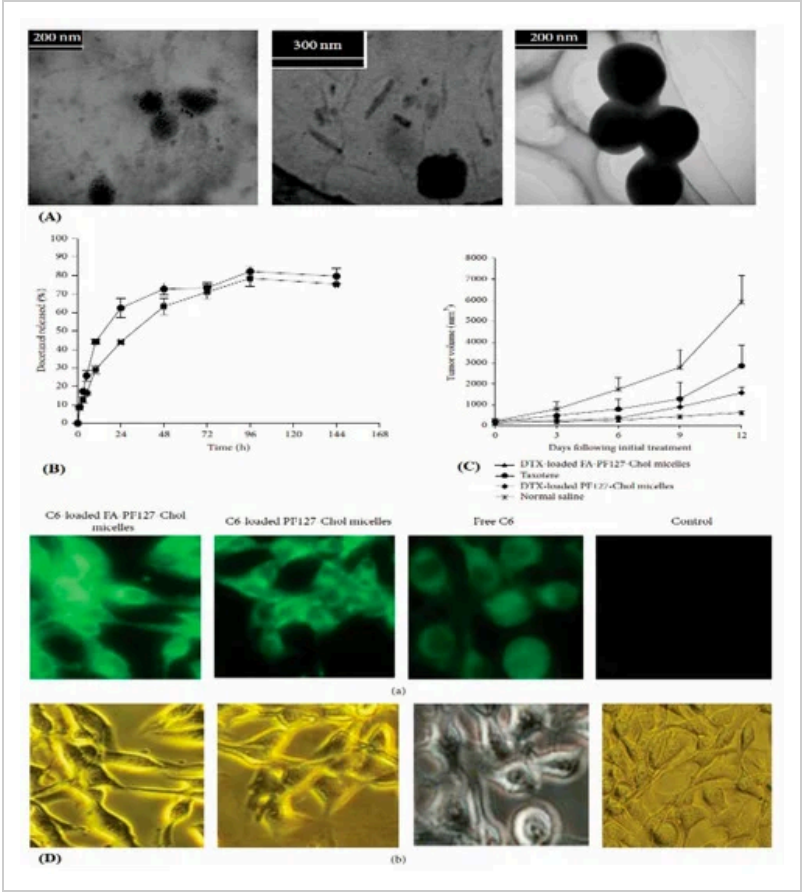


Figure 2. Illustration of (**A**) TEM analysis; (**B**) in vitro drug release studies; (**C**) in vivo antitumor efficacy studies on B16F10 melanoma bearing mice; (**D**) Results of in vitro cellular uptake studies of C6 loaded FA-targeted PF127 Chol micelles, C6 loaded non-targeted PF127 Chol micelles, free C6, and culture media (Control) via (a) fluorescent microscopy and (b) visible light microscopy. Reproduced from Varshosaz et al., 2015 [**35**] under creative commons CC BY license © 2015, The Author(s).

Chida et al., fabricated epirubicin (EPI) loaded polymeric micelles armed with pH-sensitive drug release characteristics in contradiction to axillary lymph node metastasis (ALNM) of triple-negative breast cancer (TNBC) for improving the therapeutic effect. By accumulating selectively and penetrating both the vascularized ALNM and primary tumor, as well as effective drug trigger caused by intratumoral acidic atmosphere, the proposed formulation successfully prevented the growth of ALNM and the expansion of the primary tumor [**36**].

Bae et al., designed environment-sensitive supramolecular structures for intracellular drug delivery of Adriamycin (ADR) via linking it to the polymer by a pH-sensitive coupler. The micelles treated carcinoma cells via their transportation into a cell by endocytosis, consequently avoiding cell-membrane transporters [**37**].

2.3. Polymersomes

Polymersomes are considered the next era of nanomedicine for clinical diagnosis and therapies due to their potential for controlling structure and features. These are the most favorable tools for a biomedical utility such as diagnosis and controlled drug delivery due to the compartmentalization of polymersomes. These are engineered amphiphilic block copolymers or vesicles, but in this circumstance they contain structures made up of one or more bilayers enclosing an aqueous center. In all conditions, the active load of molecules can be dissolved, dispersed, or chemically bound to nanocarriers. Their size is an important parameter for intracellular uptake. They offer better mechanical features and provide better structural and colloidal stability than liposomes in aqueous media [**38**]. Their flow properties, cellular uptake, and immune regulation depend upon their shape since their shape affects interaction with cells. As a result, being able to precisely rearrange a polymersome and comprehending the mechanisms underlying the various forms of interconversions is critical [**39**]. These polymers are amphiphilic in aqueous solvents and organized so that their hydrophilic head groups point toward the aqueous phase while their lipophilic tails are confined within the membrane's core. The phase characteristic of a carrier in a solvent determines whether it can be built into a membrane [**40**].

Polymermerosomes are presently being studied for the delivery of different probes for imaging target tissues/organs and cytotoxic medications to canceous cells and also for gene therapy [**41**]. Zavvar and co-workers developed gadolinium-based quantum dots (QDs) of targeted PEG-PCL nanopolymersomes as a diagnostic agent and doxorubicin (DOX) as an anti-cancer agent. The controlled release of encapsulated DOX was revealed in the drug release study and the formulation was found to be stable in physiologic conditions. Enhancement in both toxicity and cellular uptake ($p < 0.05$) was revealed in MTT and flow cytometry results. Overall, the study demonstrated the theranostic application of targeted polymersomes with minimization of side effects [**42**]. Zhou et al., developed polymersomes based on photo-response for delivery of doxorubicin hydrochloride. The drug can be entrapped into their hydrophilic voids and their photo-responsive disintegration resulted in a controlled release of the drug. The cellular assay demonstrated their active targeting of folic acid and photo-activated release with higher cytotoxicity of HeLa cells. The study revealed the potential of polymersomes as effective and intelligent drug carriers [**43**].

Li et al., designed paclitaxel-loaded vesicles from matrix metalloproteinase (MMP)-cleavable peptide-linked triblock copolymer, polyethylene glycol-GPLGVRG-b-poly(ε-caprolactone)-b-poly(3-guanidinopropyl methacrylamide) that showed considerably improved cellular internalization effectiveness (~10 folds) in contrast to its pure form. The developed vesicles exhibited greater cytotoxicity against MMP-overexpressing HT1080 cells and multicellular spheroids. When MMP-2 was used for treatment it resulted in cleavage of stealthy PEG shell. The vesicles undergo morphological transformation into fused multicavity vesicles and small nanoparticles, accompanied by a redistribution of PGPMA segments with 76% exposed to the outside [**44**].

To increase chemical reaction efficacy, Li et al., created polymeric nanoreactors (NRs). They created therapeutic vesicular NRs (theraNR) that were loaded with glucose oxidase (GOD). When injected into normal tissues, the theraNR was inactive. The tumor acidity activates them precisely at the target location. They effectively destroy cancer cells by inhibiting their antioxidant capacity and perfusing tumors through a synergistic action [**45**].

Li et al., created a polymersome nanoreactor based on a block copolymer prodrug that may enable new cancer oxidation/chemotherapy via selective activation at tumor locations. GOD-loaded polymersome nanoreactors were created by optimizing block copolymers to self-accumulate into polymersomes in an aqueous solution for encapsulation of GOD. Through high tumor oxidative stress, oxidation/chemotherapy and released CPT medicines synergistically killed cancerous cells and inhibited tumor development [**46**].

By simply combining a couple of differently charged block copolymers (poly(amino acid)s and PEG) in an aqueous medium, Koide et al., produced a PICsome (a novel form of polymer vesicle along with a polyion complex (PIC) membrane)

with a size range of up to 10 μm. Semipermeable characteristic of PICsomes membrane was discovered using spectral analysis and confocal laser scanning microscopic imaging. Even in the existence of serum proteins, the PICsomes maintained a high level of physiological stability, indicating that they may be used in medical applications such as therapeutic vehicles [**47**].

By incorporating stimuli-sensitive linkers into a crosslinking membrane network, Li et al., created responsive nano-reactors built on polyion complex vesicles. GOD could be protected by the fabricated ROS-responsive nano-reactor with self-improving catalytic glucose oxidation, which is attributed to stimuli-reactive vesicle development short of breakage and size-selective payload release pattern, to attain cytocidal role by oxidative stress induction and glucose starvation. Pyroptosis, an immunostimulatory type of cell demise, was produced by the GOD-loaded therapeutic nanoreactor [**48**].

2.4. Dendrimers

Dendrimers (accurately designed, highly-radial polymeric frameworks) were incorporated as promising drug nanocarriers, although some authors seem to be more satisfactory in their description as “polymer therapeutics” [**49**]. These are polymeric nanomaterials having 3-D macromolecule having branch-like shape, with less than 15 nm of size and coming out from a central portion [**50**,**51**].

Pillay et al., developed folic acid loaded dendrimer-functionalized selenium nanoparticles (FA-PAMAM-SeNPs) with size <150 nm and zeta potential of >25 mV. The developed formulation demonstrated greater cell viability (>85%) in contrast to placebo nanocomplexes (75%), confirming the importance of selenium in the developed formulation. The developed formulation resulted in greater overall transgene expression that showed their improved receptor-mediated cellular uptake [**52**].

Bartusik-Aebisher et al., developed a trastuzumab-dendrimer-fluorine DDS in which API was attached to dendrimers (fluorinated) via covalent bonding. They found that the potential of the developed formulation was more efficient than pure trastuzumab [**53**].

Mota et al., encapsulated l-buthionine sulfoximine in folate-targeted polyurea dendrimers. Initially, the burst release was observed that was expected in these delivery systems. After 1 h, about 60% of drug release was found, reaching a plateau after 3 h. After 24 h, 90% drug release was found [**54**].

Zhang et al., 2022 fabricated a third generation of poly(phosphorhydratzone) radical dendrimers and reported their action against the diagnosis of brain tumors [**55**]. It was also reported that the developed agent did not cause any toxicity to the cells.

Knauer et al., 2022 designed a polycationic phosphorous dendrimer-based approach for the delivery of siRNA to target Lyn [**56**].

2.5. Nanosized Hydrogels

Recently, the advancement of biological science managed novel developments in the area of in vitro cell culturing. Various novel innovations such as cells on chips/cell microarray need consistent supportive materials with decent biocompatibility, and cell adhesion and are simple to use [**57**]. Nanomaterial-based systemic drug vehicles need various properties particularly delivery of drugs to targeted sites. These parameters increase ligand conjugation and passive targeting, resulting in an improvement in active targeting. Hydrogel-medicated local DDSs directly deliver drug moieties at the target site without complications due to their implantation proximate to carcinoma tissues. It has been already found that a visible light-cured injectable glycol chitosan (GC) hydrogel-based local DDS can be employed for an ailment of various solid cancers [**58**]. Nanosized hydrogels developed by physically or chemically cross-linking polymer systems and commonly called nanogels can also be known as nanocarriers based on polymers [**59**]. Hydrogels may be custom-shaped and thickened, and their surfaces can be shaped using lithography methods. Furthermore, hydrogels can be fabricated with biomolecules to achieve unique characteristics [**58**].

Despite their solute permeability and controllable release features, hydrogels are efficient carriers for medicines, proteins, and others. Hydrogels made of chitosan can create multilayered structures, which is why they demonstrate utility in distributing bioactive molecules including insulin and growth factors, and in cell and tissue organization. They can be administered to the body using very simple, minimal stress procedures for the administration such as injection and both ocular and nasal administration [**60**].

2.6. Polymeric Cubosomes

Polymeric cubosomes are newly discovered inverse bicontinuous cubic mesophases. These are colloidal particles containing mesoporous block copolymers having well-defined reticulated pore networks. Kim et al., prepared polymer cubosomes using block copolymer which was synthesized by linking hydrophilic PEG and hydrophobic polyisoprene polymer blocks. The cubosomes were prepared via solution self-assembly of block copolymers [**61**]. The block copolymers with non-linear structures undergo self-assembly to form inverse mesophases in solution [**62**]. These are highly efficient nanoparticles that are developed from a lipidic cubic state and protected by an outer layer of polymer. The polymeric cubosomes are considered to have improved endurance and have a greater capacity to surround and embody hydrophobic anticancer agents due to their liquid crystalline membrane structure [**63**,**64**,**65**,**66**,**67**]. As a result of recent developments, applications involving

drug delivery, membrane bioreactor, artificial cells and biosensors can be developed in vitro in both bulk and nanoparticle formulations [**68**].

Zhang et al., developed the cubosomes loaded with a combination of anti-cancer drug cisplatin and paclitaxel for an effective targeting against cancer cells. In this work, a slow and sustained release was obtained by coating the cubosomes with poly-Ɛ-lysine. The coating prevented the initial burst of API resulting in a sustained drug release. TEM studies revealed very easily distinguishable spherical polyangular structures. Both initial reductions in burst discharge of drug and a slow, sustained discharge with time were revealed by in vitro studies. The cytotoxicity studies revealed the non-toxic behavior of cubosomes [**69**]. The morphological pattern, in vitro drug release, and cell viability investigations are shown in **Figure 3**. The impedance measurement and fluorescent imaging studies further confirmed their therapeutic efficiency against HeLa cells. Overall, the study affirms their significance as an effective nano-drug carrier.

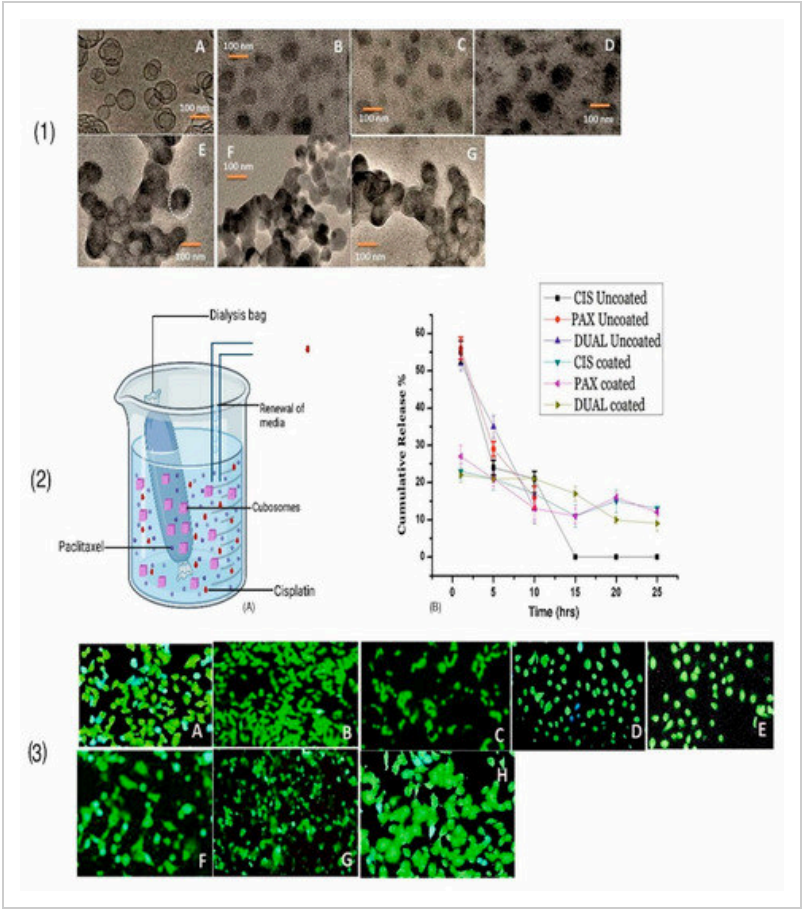


Figure 3. Illustration of (**1**) morphological analysis of (**A**) blank cubosomes, (**B**) uncoated CIS, (**C**) uncoated PAX, (**D**) uncoated DUAL, (**E**) coated CIS, (**F**) coated PAX and (**G**) coated DUAL cubosomes; (**2**) in vitro drug release studies (**A**) assembly and (**B**) comparative results of drug release; and (**3**) Cell viability studies on exposure of Human hepatoma HepG2 cells to (**A**) PBS blank cubosomes, (**B**) control cubosomes, (**C**) coated CIS, (**D**) uncoated CIS, (**E**) coated PAX, (**F**) uncoated PAX and (**G**) coated DUAL, (**H**) uncoated DUAL cubosomes. Reproduced from Zhang et al., 2020 [**69**] under creative commons CC BY license © 2020, The Author(s).

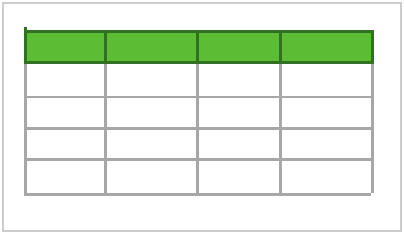
Boge and co-workers developed the cubosomes for application in the delivery of peptide LL-37 for the ailment of skin infections. In the first strategy of development, the LL-37 was first added into a gel followed by dispersion into nanoparticles. The next strategy involved the adsorbing LL-37 onto preformed cubosomes. The last strategy consisted of the incorporation of LL-37 during their spontaneous formation in an ethanol/glycerol monooleate mixture. The pre-loaded cubosomes showed maximum efficiency in killing the bacteria [**70**].

3. Routes of Administration and Applications of Polymer Based Nanocarriers

The merging of polymer technology with pharmaceutical research resulted in a quantum leap in terms of innovation in designing and developing novel DDSs such as nanotechnology-based delivery systems. The application of nanopolymers in pharmaceutical and biomedical fields are swiftly growing, such as developing scaffold in tissue engineering, designing a drug delivery system, ophthalmology, dentistry, bone repair, implantation of medical devices, prosthesis, and many other medical fields. It is possible to obtain desirable properties in a pharmaceutical polymer by modifying its physical and chemical characteristics such as molecular weight, type, composition, co-polymerization, biomimetic, and co-processed excipient [**71**].

The discovery of the first synthetic drug delivery system sparked an interest in designing novel biodegradable polymers to replace the non-degradable polymer. The advent of bioadhesive polymer resulted in improved residence time and intimate contact between polymer and epithelial surface. Further advancements in polymer science led to the discovery of smart polymers that release the drug in presence of specific stimuli (physical, chemical, or biological) [**72**]. These nanopolymers were widely scrutinized based on their mechanism for controlled release of drugs and the understanding of their behavior in solid and liquid dosage forms, disperse systems, transdermal patches, implants, and many others [**73**]. There are numerous biodegradable and non-biodegradable polymers possessing different physicochemical characteristics used in pharmaceutical and biomedical fields (as shown in **Table 1**) [**74**,**75**,**76**,**77**,**78**,**79**,**80**,**81**,**82**,**83**,**84**].

Table 1. Applications of nanopolymers in pharmaceutical and clinical medicine.



Drug molecules (such as proteins, peptides, and DNA) are protected and stabilized by these polymers from environmental hazard degradation. Even though polymers have wide applications in the pharmaceutical industry, the criteria for proper selection and design of polymers is a challenging task and requires an in-depth understanding of its bulk properties (such as molecular weight, diffusion, and dissolution-controlled release based on solubility) and surface properties (such as surface energy, smoothness, hydrophilicity, lubricity, water sorption, and swellability) in addition to its physicochemical properties. The structure-activity relationship and polymeric matrices can be altered by changes in chemical composition and microstructural design [**71**]. The polymer-based nanocarriers are widely employed in various arenas for differenet routes that are discussed in brief below.

3.1. Applications of Polymeric Nanocarriers in Oral Drug Delivery

The oral route has been explored as a non-invasive and preferred delivery route for numerous therapeutic agents. However, oral delivery suffers poor bioavailability and enzymatic instability [**85**,**86**,**87**]. Modulation of gastrointestinal transit time is a challenge that can be overcome by increasing the residence time of drugs within the gastrointestinal tract. Polymeric nanocarriers emerged as prominent oral delivery vehicles owing to their interesting bioavailability, superior drug entrapment, control release, and minimal toxicity. The improved oral bioavailability of polymeric nanocarriers is due to the enhanced mucoadhesion property of polymer that sticks to the mucosa of the gastrointestinal tract leading to prolonged gastro-intestinal transit time and thereby enhancing mucosal penetration (as shown in **Figure 4**).

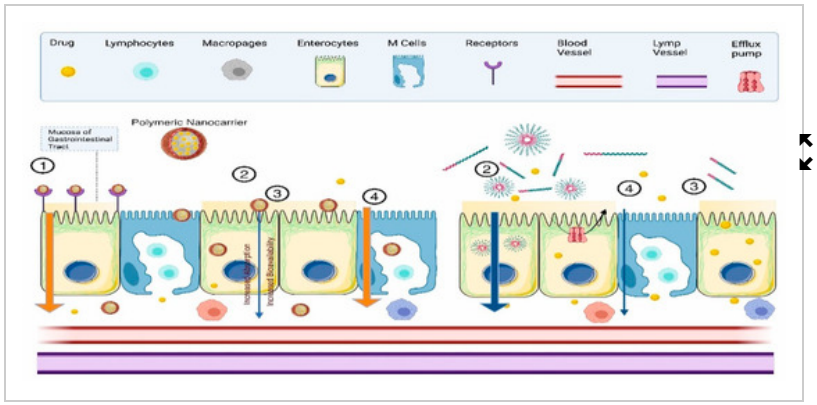


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Figure 4. Illustration of mucoadhesion property of polymeric nanoparticles (**right**) and micelles (**left**) in gastrointestinal transit time modulation resulting in enhanced mucosal penetration and oral bioavailability. ① receptor-mediated endocytosis, ② transcellular transport, ③ paracellular transport, and ④ M cell-mediated transport.

Polymeric materials being biocompatible, biodegradable, non-immunogenic, and functional that offer numerous advantages for drug delivery such as active drug targeting via surface functionalization and controlled release in the body [**88**]. Polymers-based nanocarriers have been extensively explored as vehicles for oral delivery of various peptides, genes, medicaments, proteins, siRNA, probiotics and vaccines as enlisted in **Table 2** [**89**,**90**,**91**,**92**,**93**,**94**,**95**,**96**,**97**,**98**,**99**,**100**,**101**,**102**,**103**,**104**,**105**,**106**,**107**,**108**,**109**,**110**]. Polylactic acid (PLA), poly-glycolic acid (PGA), polycyanoacrylates (PCA), polyethyleneimine, and polycaprolactone are the main synthetic polymers widely employed for oral drug delivery. Chitosan, dextran, gelatin, alginate, and agar among which chitosan is most widely used owing to its mucoadhesive, biocompatible, and non-toxic properties [**100**].

Table 2. Various nanopolymers employed as nanocarriers for oral delivery of drugs, peptides, and genes.

3.2. Applications of Polymeric Nanocarriers in Ocular Drug Delivery

Several efforts have been made in ocular drug delivery to increase corneal assimilation by improving the drug residence time with the ocular surface through bioadhesive polymers [**111**]. The precorneal fluid contains a mucin-glycocalyx domain which is bound to the corneal surface and serves as a substrate for adequate binding of polymeric chains present in ocular formulation through non-covalent bonding resulting in prolonged drug contact time with the ocular surface and thereby improved bioavailability (as demonstrated in **Figure 5**). The widely used polymers for ocular drug delivery include chitosan, gelatin, alginate, synthetic poly-alkyl cyanoacrylate, poly-ε-caprolactone, poly-lactic acid, poly-lactic-co-glycolic acid, and polystyrene [**112**]. The biological properties of the polymer such as biodegradability, non-toxicity, biocompatibility, and mucoadhesiveness resulted in the development of various colloidal vehicles for ocular routes such as polymeric nanoparticles and polymeric micelles [**113**]. The positive surface charge of polymeric nanocarriers allows prolonged contact time of API on the ocular surface due to increased interaction with glycoprotein of cornea and conjunctiva leading to the formation of a precorneal depot that is responsible for the prolonged release of API.

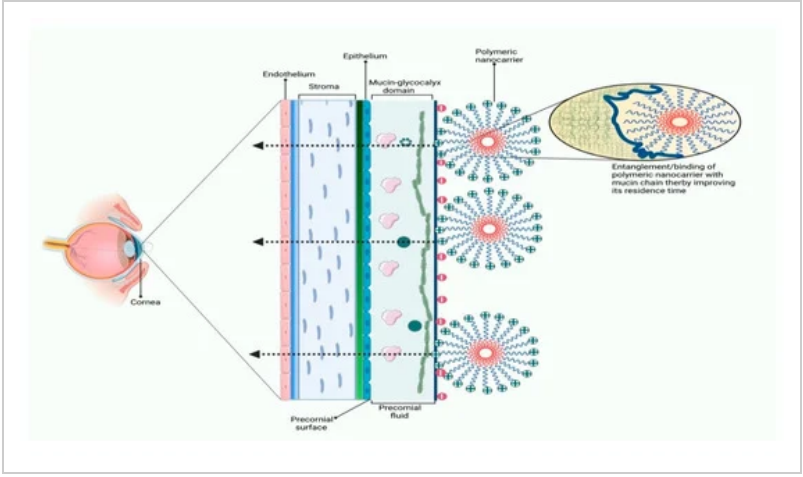
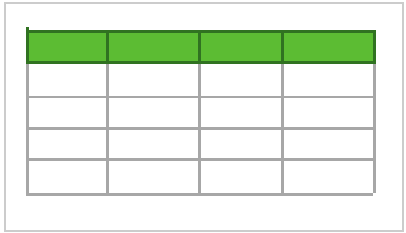


Figure 5. Illustration of ionic interaction of polymeric nanocarriers with corneal glycocalyx domain resulting in increased ocular surface contact and improved drug penetration.

Sharma et al., formulated polymeric nanoparticles using bioadhesive positively charged Eudragit® RS100 and Eudragit® RL100 which are official by USFDA as excipients for controlled drug delivery. The presence of a positive charge on

nanoparticles resulted in ionic interaction with anionic mucin existing in the mucus layer of tear film leading to sustained release of drug and improved ocular penetration [**114**]. Mahor et al. fabricated moxifloxacin-loaded polymeric nanoparticles using positively charged gelatin for its effective ocular delivery and controlled release in the corneal eye layer [**115**]. Mittal et al. utilized chitosan, a polycationic bioadhesive polymer, to develop timolol maleate-loaded polymeric nanoparticles. The polymeric nanoparticles exhibited significant bioadhesive strength due to the existence of polymeric chains and polar functional groups in chitosan. The in vitro experiment displayed burst release (27.18% in 1 h) and then sustained release (90.55%) up to 24 h. The ex vivo transcorneal permeation investigation exhibited appreciable corneal penetration of timolol from polymeric nanoparticles compared to conventional eye drops attributable to the penetration-enhancing property of chitosan [**116**]. **Table 3** compiles significant reports on the ocular delivery of polymeric nanocarriers [**117**,**118**,**119**,**120**,**121**,**122**,**123**,**124**,**125**,**126**,**127**,**128**,**129**,**130**,**131**,**132**,**133**,**134**,**135**,**136**,**137**,**138**,**139**,**140**,**141**,**142**,**143**,**144**,**145**,**146**,**147**,**148**].

Table 3. A cross-section report on polymers employed as nanocarriers for ocular drug delivery.



3.3. Applications of Polymeric Nanocarriers in Parenteral Drug Delivery

This is the preferred route for delivery of active ingredients having a narrow therapeutic index and poor bioavailability. However, intramuscular and subcutaneous injection dosage form suffers rapid drug elimination. Continuous *IV* infusion is one of the approaches for maintaining constant drug delivery which also avoids hepatic metabolism but the patient has to be hospitalized. Among methodologies to overcome traditional medical treatment, simulation of *IV* infusion by use of parenteral controlled release formulations has gained interest. **Figure 6** depicts sustained and targeted delivery of drugs via polymeric nanocarriers following parenteral administration [**148**].

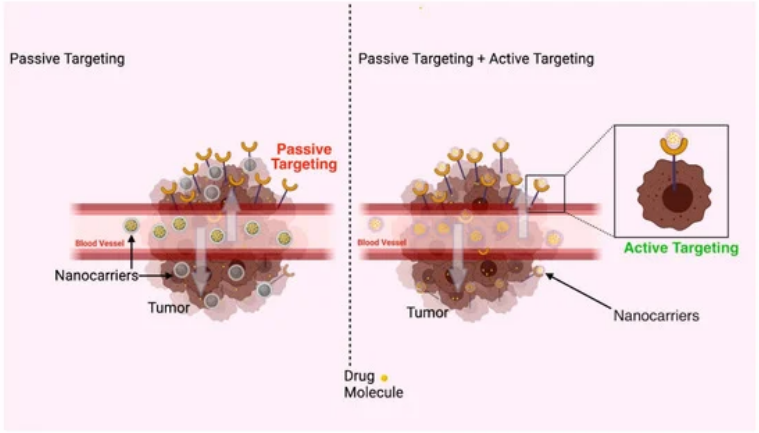


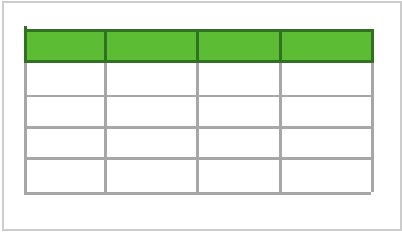
Figure 6. Graphical representation of active and passive targeting of drugs via polymeric nanocarriers following parenteral administration.

Active targeting can be achieved at different levels depending on the extent of penetration; it may occur at the organ, cell, or subcellular level. It is important to note that in solid tumors, even active targeting processes begin with the passive accumulation of the DDS in the tumor tissue, so any actively targeted carrier must satisfy the basic requirements outlined for passively targeted systems: the polymer/nanoparticle should be biocompatible, and the system must be stable in circulation, long-circulating, and of a size that permits efficient extravasation and accumulation in tumors. In addition, actively targeted systems are often more effective in general than passively targeted alternatives [**149**].

Intravenous administration of biodegradable polymeric nanocarriers has been effectively employed for controlling and targeting drugs to a specific site of action. In particular, biodegradable nanocarriers formulated from PLGA have been extensively studied for sustained and targeted delivery of numerous agents [**150**]. It is a copolymer synthesized by co-polymerization of two different monomeric units, the cyclic dimers of glycolic acid and lactic acid, linked through an ester linkage. PLGA has been approved by FDA and European Medicine Agency for usage in parenteral DDSs due to its biocompatibility and biodegradability. Several forms of controlled DDSs have been studied among which polymeric nanocarriers have gained attention for diagnostics, prognostics, controlled and sustained delivery of protein, peptide, pDNA, and other therapeutic agents owing to their biocompatibility, non-immunogenicity, and several others are enlisted in **Table 4** [**151**,**152**,**153**,**154**,**155**,**156**,**157**,**158**,**159**,**160**,**161**]. Natural and synthetic polymers have been extensively investigated as

carriers for several anticancer drugs. Conjugation of drugs with these polymeric carriers increases their circulation time in the blood leading to passive gathering in tumor tissues due to the enhanced permeability and retention (EPR) effect [**140**,**141**].

Table 4. A summary of reports on polymers employed as nanocarriers for parenteral delivery of drugs, peptides, and genes.

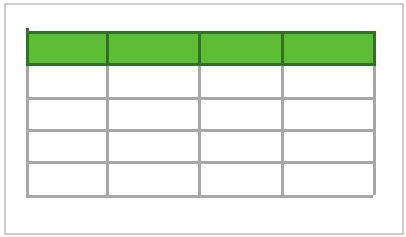


3.4. Applications of Polymeric Nanocarriers in Topical Drug Delivery

Polymer-based nanocarriers have been extensively explored as topical formulations to improve cutaneous delivery owing to their physicochemical attributes which prevent degradation or denaturation of unstable drugs, reduce their toxic side effects through controlled release, and enhance their cutaneous penetration across the biological membrane more precisely skin barrier due to their ability to increase the concentration gradient [**162**]. Polymers serve an important role in the preparation of polymeric nanocarriers which might be natural or synthetic. The extensively used natural polymers are chitosan, alginate, gelatin, and albumin. Synthetic polymers include polylactides, PVA, poly(acrylic acid), polyacrylamide, PEG, and several others. These polymers have gained attention as a substrate for colloidal vehicles due to their blood stability, non-toxicity, non-thrombogenic, non-immunogenic, and non-inflammatory characteristics [**163**,**164**].

Natural polymers tend to develop hydrogels which render them suitable carriers for peptides, oligonucleotides, proteins, and water-soluble APIs for improving the absorption and targeted drug delivery of polymeric nanocarriers for topical administration. Chitosan-based nanocarriers have been widely used among the natural polymers for topical delivery. An N-deacetylated derivative of chitin, chitosan is a cationic polymer that is made up of glucosamine units. Its additional anti-oxidant, anti-inflammatory, and anti-microbial features make it an appropriate carrier for therapeutic delivery. **Table 5** reports a compilation of polymeric nanocarriers for topical delivery of drugs, peptides, and genes [**148**,**149**,**150**,**151**,**152**,**153**,**154**,**155**,**156**,**157**,**158**,**159**,**160**,**161**,**162**,**163**,**164**,**165**].

Table 5. A cross-section of reports on polymers employed as nanocarriers for topical delivery of drugs, peptides, and genes.

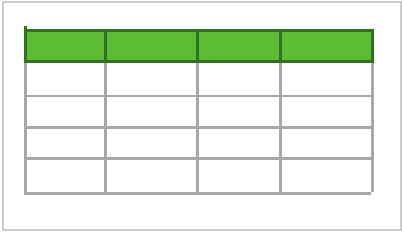


4. Practical and Regulatory Consideration

Nanomedicine is a new area integrating nanotechnology with pharmaceutical and biomedical sciences, intending for the development of medicines with improved safety and toxicological profiles [**171**]. In drug formulation, nanoparticles can impart several physical and biological advantages such as improved pharmacokinetic profile, enhanced tissue/organ selectivity, and decreased toxicity as compared to conventional medicines [**172**]. FDA governs the pre-market approval process of conventional drugs, biologics, and medical devices, and likewise, nanomedicines are also subject to a normal range of pre-clinical and clinical validation. Based on available literature and the Centre for Drug Evaluation and Research (CDER), the no. of clinical trials involving nanomedicines has increased three-fold and the utility of nanomaterials in these products has incredibly risen in the past two decades [**173**]. Based on previously reported results, liposomes account for the largest proportion of therapeutic nanoproducts with over 33%, followed by nanocrystals and emulsions which accounts for 23% and 14%, respectively. Nine percent of products used include polymer ion complexes while micelles have a 6% share. The studies also show that specific nanostructures (quantum dots, carbon nanotubes, graphene) also have a small percentage of shares in these products [**174**].

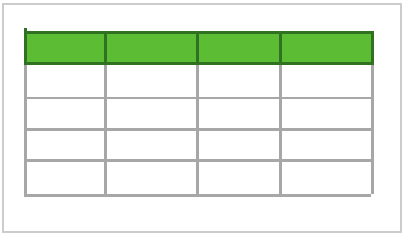
In nanomedicine, polymeric nanoparticles serve two purposes (i.e., controlled release of drug-using biodegradable polymers and improved half-life and bioavailability by forming drug-polymer conjugates) [**175**]. Copaxone and Neulasta are two polymeric drugs out of the top 10 bestselling drugs in the US in 2013 [**176**]. There are several polymeric immobilized nanomedicines as shown in **Table 6** that are approved by USFDA [**177**,**178**,**179**,**180**,**181**,**182**,**183**,**184**].

Table 6. USFDA-approved polymeric products.



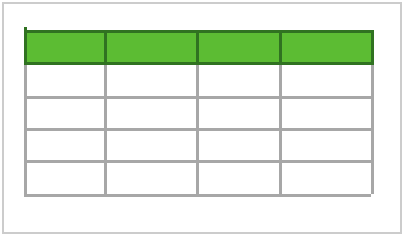
The composition and structure of polymeric micelles can be precisely modified to achieve specific release characteristics and drug loading. The only approved polymeric micelle is a traditional formulation of oestradiol (Estrasorb™). The use of modern co-block polymers led to the development of polymeric micelles at a lower critical micellar concentration with high stability [**185**]. Few polymeric micelles formulations that are in late-stage clinical trials, especially *i*/*v* administered formulations are shown in **Table 7** [**186**].

Table 7. A cross-section of reports on polymeric nanocarriers under clinical trials.



The various patented reported polymeric nanocarriers intended for oral, ocular, topical, and parenteral administration are enlisted in **Table 8** [**187**].

Table 8. A random sample of patent reports on polymeric nanocarriers intended for oral, ocular, topical, and parenteral administration.



4.1. Toxicity Issues

Most of the polymeric nanocarriers are generally regarded as safe owing to the interesting physicochemical characteristics of the polymer which render them biocompatible and biodegradable. Copolymers PLA and PLGA acid are the most extensively employed biomaterials for polymeric nanocarriers, that undergo hydrolytic degradation within the body leading to the generation of endogenous lactic acid and glycolic acid monomers which are metabolized via Kreb's cycle leading to minimal systemic toxicity [**188**]. However, various factors could affect the toxicity of the polymeric nanocarriers not limited to their surface chemistry, size, and concentration as reported by Ahlawat and Henriquez [**189**]. The size of the polymeric nanocarriers significantly affects the cellular uptake and hence causes toxicity. Enhanced cellular uptake of polymeric nanocarriers leads to increased interaction with intracellular components which triggers the production of free radicals, cytokines, and reactive oxygen species (ROS) causing organelle destruction and finally cell lysis that leads to toxicity issues [**190**,**191**].

Grabowski and Hillaireau studied the toxicity of surface-mediated polymeric nanoparticles on human-like THP-1 macrophages wherein cell mitochondrial activity, apoptosis detection, ROS, and pro-inflammatory cytokines production were chosen as toxicity criteria. Positively, negatively and neutral PLGA nanoparticles were fabricated with chitosan, poloxamer 188, and PVA stabilizers. The stabilizer-free PLGA nanoparticles did not exhibit any sign of toxicity at or above a therapeutic concentration of 0.1 mg/mL. However, PLGA nanoparticles prepared using stabilizers exhibited cytotoxicity above the therapeutic level of 0.1 mg/mL, thereby confirming the safety of the polymeric nanocarriers and the toxicological contribution of stabilizers used in the polymeric nanocarrier formulation [**192**].

Rejinold and Muthunarayanan reported non-toxicity of the chitosan-g-poly-N-vinyl caprolactone nanoparticles in an array of cell lines at 100–1000 µg/mL [**193**]. Similarly, Dandekar and Dhumal reported the cellular safety of the Eudragit® S100 polymeric nanoparticles through genotoxicity studies [**194**]. Singh and Ramarao examined the concentration-dependent toxicity of PLGA, PLA, poly-ϵ-caprolactone, and poly-lactide-caprolactone nanoparticles using macrophage (RAW 264.6), hepatocytes (Hep G2), lung (A549), kidney (A498) and neuronal epithelial (Neuro A2) [**180**]. Polymeric nanoparticles did not display toxicity at 100 µg/mL concentration as marked by no change in cell viability. However, a reduction in cell viability was detected at 300–1000 µg/mL concentration after 72 h incubation of polymeric nanoparticles with cell lines.

4.2. Effects of the Route of Administration on Bioavailability

Polymeric nanocarriers have become a prominent field of research in the arena of drug delivery since they can improve pharmacokinetic and bioavailability that results in effectiveness of various drugs, enhanced hydrophilicity, reduction of

interaction with plasma and cellular proteins, and greater desposition of drug at target sites [**195**]. Oral, intravenous, intramuscular, intranasal, intradermal, and transdermal administration are the foremost delivery routes for drugs. Other routes, such as ocular delivery, have also been developed for localized and targeted drug delivery, ocular route is employed that declines unwanted systemic side effects. Specific barriers are encountered by each route of administration route. Roughly, solubility and permeability are two major issues related to drug substances that are responsible for bioavailability upon administration [**196**]. Improved stability and bioavailability of therapeutic agents coupled with extended drug release profile and possibility to embed numerous drugs makes suitable candidate for pharmaceutical products [**197**].

Various factors govern the assimilation of the drug such as drug solubility, mucosal permeability, and stability in the GI tract milieu. Efforts are ongoing to tackle these issues have focused on physicochemical, biochemical, biological, and metabolic barriers that are responsible for limited assimilation. Different pharmaceutical technologies and DDSs such as nanocarriers, micelles, cyclodextrins, and lipid-based carriers have been discovered for improving the assimilation of a drug.

GI tract leads to experience a varied range of pH, enzymatic degradation and low permeability across the membrane. Polymeric NPs may be an auspicious strategy that can improve stability to crossing the mucus barrier or interact with a mucus layer and increase residence and contact time [**198**].

Oral, aerosol, and nasal vaccination is a novel immunization technique that employ nanocarriers to administer vaccine. Therapeutic agents and vaccines with lower penetration potential can be administer via transmucosal route while maintaining their biological activity. Nowdays, nanocarriers for transmucosal delivery are gaining more attention since delivery via oral mucosa helps evade first-pass metabolism, improves bioavailability and acts as a means of rapid drug transport to the systemic circulation. The oral transmucosal vaccine delivery by nanocarriers is the most upcoming innovation in efficient vaccine delivery [**199**,**200**].

5. Future Prospects

Over the past few decades, polymer science has evolved to mediate safe and efficient drug delivery for the ailment of several health conditions. Over the years, the usage of polymers has been limited to oral delivery, but critical advances in polymer science such as the development of biodegradable nanosized carriers have accelerated their use in parenteral formulations. Further innovations in the application of polymers have led to a more sophisticated approach, thereby improving the pharmacokinetics, decreased toxicity, stealth effect, cell or organ-specific targeting, and controlled release of the drugs;

these innovatioms have made it possible to administer two or more drugs or imaging/diagnostic agents within the same drug delivery system, leading to more effective therapy [**198**,**201**,**202**]. Due to the development of novel polymers with highly complex structures and functions, its scope has widened in the field of nanoformulation, particularly in imaging and theranostic applications [**203**]. The recent trend in polymer science is leaping from the traditional linear polymer to the complex hyperbranched or hybrid polymers.

New technologies and advances in the synthesis and manufacturing process such as 3-D printing, supercritical fluid technology, and nanoengineering are revolutionizing polymer-based drug delivery systems [**204**]. Scale-up in the field of nanotechnology has always been troublesome due to non-reproducibility between batches and future evolution is expected from the introduction of new types of polymers also known as smart polymers (stimuli-responsive, enzyme responsive) that fulfill requirements to be used as a constituent in drug delivery systems [**205**]. Nevertheless, future development should progress towards understanding the interaction of polymers or polymercarriers with the biological system. Most studies carried out in the present scenario neglect the biocompatibility studies that fail new devices at the later stage of development. Adequate animal models and in vitro studies can lead to a breakthrough in polymer science as safe and effective platforms for drug delivery [**206**].

Nanomedicine has evolved over the last several decades from biologically inert substances to increasingly intelligent systems aiming at improving in vivo functioning. But, we must acknowledge that most systems depend on logical explanations-including some over-explanation rather than conclusive proof, which is a pivotal event in nanomedicine's progress. The importance of investigating nano-bio interactions and desired functioning at the molecular, cellular, and tissue levels is often ignored [**34**].

An overall change in global markets, technological innovations, and data on the pathophysiological and cell-based mechanisms should be considered that can outline the future progress for polymers in drug delivery.

6. Conclusions

Polymeric nanocarriers have a great potential for clinical applications. They are expected to perform both diagnostic and therapeutic functions due to their interesting physiochemical characteristics which offer numerous advantages such as biocompatibility, biodegradation, non-immunogenicity, non-inflammatory, low toxicity, prolonged drug release, and active site targeting, rendering them safe for therapeutic applications at a certain concentration. At present, few polymeric systems can be

employed for diagnostic and therapeutic applications. Among nanomaterials, polymeric nanocarriers have been the exceedingly explored delivery vehicles due to their wide range of solubility (hydrophilic and lipophilic surfaces) and self-assembling properties. They offer solubilization of hydrophobic or poorly soluble molecules, sustained drug release, and protection to encapsulated drugs from degradation and metabolism. The polymeric nanocarriers are considered as the best ones due to their good drug loading capacities, biodegradability, and stimuli-responsive control.

Polymeric nanocarriers are safe for the parenteral route of administration than their surfactant counterparts. They are kinetically stable and hence dissociate very slowly in blood, resulting in extended circulation time. Recently biopolymeric nanocarriers have immerged as storms which are developed using polymers obtained from natural sources. These biopolymeric nanocarriers offer better biodegradability, biocompatibility and could be the next generation nanocarriers for drug delivery. In the literature, there are several studies for developing new generation polymeric nanocarriers to obtain future smart and multifunctional nanomedicine.

Polymeric nanocarriers have many challenges for a carrier system such as biocompatibility, biodistribution, side effects, and biological barriers. Their biodegradability and biocompatibility profiles should be critically investigated before utilization in human clinical trials. One major concern that requires immediate attention is the regulatory concerns regarding the approval of polymer-based carriers since establishing new guidelines and policies seem to be in the initial stages of development. New criteria for manufacturing, testing, and storage of these nanosized polymeric materials should be defined by the regulatory agencies [**207**]. So, their manufacturing and regulation have challenges for their further development in various fields.

Author Contributions

A.K.T.—Conceptualization, Writing—Original Draft Preparation; S.C.U.—Writing—Review & Editing; M.K.—Conceptualization, Supervision; K.P.—Writing—Review & Editing, Data Curation; D.K.—Writing—Review & Editing, Data Curation; R.V.—Writing—Review & Editing; S.B.—Writing—Review & Editing; M.H.R.—Writing—Review & Editing. E.E.S.M. and S.C.—Writing—Review & Editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Abbreviations

DOX	Doxorubicin
NSM	Nanostructured materials
PCA	polycyanoacrylates
PCL	Polycaprolactone
PDLLA	Poly-DL-lactic acid
PEG	Polyethylene glycol
PGA	poly-glycolic acid

PLA	Polylactic acid (*l*)
PLGA	Poly-lactic-co-glycolic acid
PVA	Polyvinyl alcohol
QDs	Quantum dots
USFDA	United States Food and Drug Administration.

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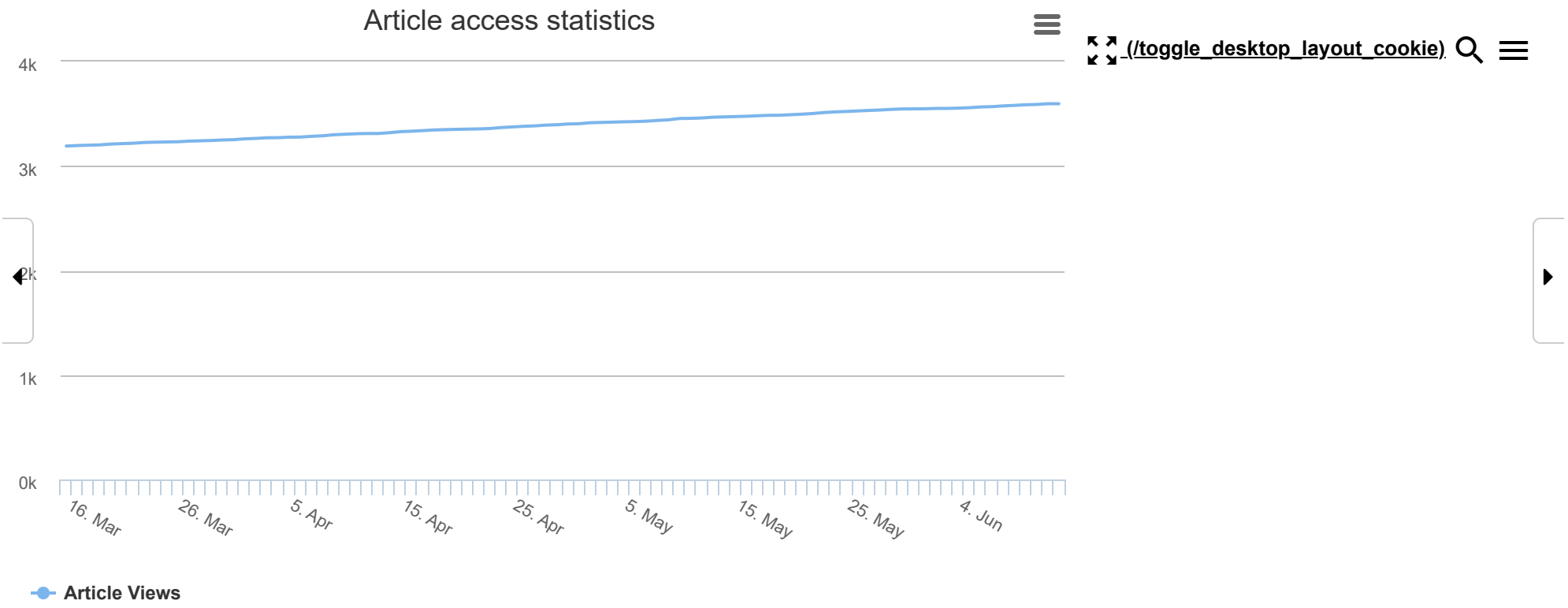

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Review

Nanocarrier-Based Approaches to Combat Chronic Obstructive Pulmonary Disease

Tarun Virmani, Girish Kumar, Reshu Virmani, Ashwani Sharma & Kamla Pathak

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Abstract

chronic obstructive pulmonary disease (COPD). Among these, COPD is more prominent worldwide. Various conventional approaches are available in the market for the treatment of COPD, but the delivery of drugs to the target site remains a challenge with conventional approaches. Nanocarrier-based approaches are considered the best due to their sustained release properties to the target site, smaller size, high surface-to-volume ratio, patient compliance, overcoming airway defenses and improved pharmacotherapy. This article provides updated information about the treatment of COPD along with nanocarrier-based approaches as well as the potential of gene therapy and stem cell therapy to combat the COPD.

Keywords:: chronic obstructive pulmonary disease gene therapy nanocarrier stem cell therapy

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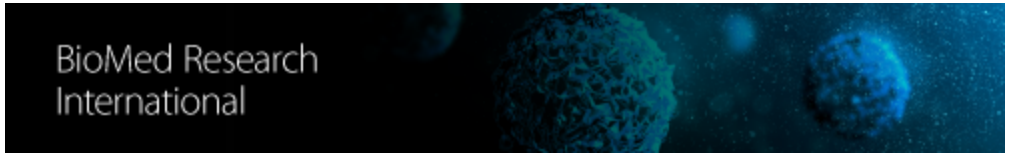

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Artificial Intelligence-Based Data-Driven Strategy to Accelerate Research, Development, and Clinical Trials of COVID Vaccine

Ashwani Sharma, [1] Tarun Virmani, [1] Vipluv Pathak, [2] Anjali Sharma, [3] Kamla Pathak,[4] Girish Kumar, [1] and Devender Pathak [5]

Abstract

The global COVID-19 (coronavirus disease 2019) pandemic, which was caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in a significant loss of human life around the world. The SARS-CoV-2 has caused significant problems to medical systems and healthcare facilities due to its unexpected global expansion. Despite all of the efforts, developing effective treatments, diagnostic techniques, and vaccinations for this unique virus is a top priority and takes a long time. However, the foremost step in vaccine development is to identify possible antigens for a vaccine. The traditional method was time taking, but after the breakthrough technology of reverse vaccinology (RV) was introduced in 2000, it drastically lowers the time needed to detect antigens ranging from 5–15 years to 1–2 years. The different RV tools work based on machine learning (ML) and artificial intelli (AI). Models based on AI and ML have shown promising solutions in accelerating the discovery and optimization of new anti effective vaccine candidates. In the present scenario, AI has been extensively used for drug and vaccine research against SAR therapy discovery. This is more useful for the identification of potential existing drugs with inhibitory human coronavirus by using

different datasets. The AI tools and computational approaches have led to speedy research and the development of a vaccine to fight against the coronavirus. Therefore, this paper suggests the role of artificial intelligence in the field of clinical trials of vaccines and clinical practices using different tools.

1. Introduction

Virus-caused infectious diseases have long been the most difficult challenge in human health. High-infectivity and high-mortality diseases are particularly feared, and in the past, people viewed them as a tragedy or disasters (Hilleman et al., [1]). Humankind has been able to overcome the irrational dread of death because of advances in recognizing the etiology of viral diseases and knowledge of microbiology, which were followed by the creation of numerous vaccines. Vaccination is often regarded as one of the greatest achievements in medical history. Immunization has saved a lot of lives, and its significance continues to expand. Despite countless efforts to develop qualified and efficient vaccinations, there are inadequate barriers in place to protect populations from diseases that could produce epidemics or pandemics (for example, the Ebola virus epidemic) (Kilbourne, [2]; Gostin et al., [3]). As a result, scientists are working to expand the viral infection that may be prevented by vaccinations, as well as the population groups that will benefit from vaccination in the long term. As of now, Coronaviruses (CoVs) are responsible for the causes of serious illnesses in humans and a variety of animal hosts, including respiratory, gastrointestinal, and systemic diseases. Infections with the CoVs have been found in cattle, swine, rats, cats, mink, dogs, bats, palm civets, horses, camels, ferrets, rabbits, snakes, and a variety of other wild mammals and bird species (Fehr and Perlman [4], Kahn and McIntosh, [5]). Since the very first outbreak in 2002, the Coronaviridae virus family, which causes pneumonia-like symptoms, has been a global danger (Khan et al., [6]). The infections including severe acute respiratory syndrome (SARS) and middle eastern respiratory syndrome (MERS), which first occurred in 2002 and 2013, respectively, caused respiratory and gastrointestinal problems (Hilgenfeld and Peiris, [7]). SARS-COV-2, which has been identified as the virus that causes COVID-19, having symptoms ranging from a common cold to serious respiratory failure, was the source of a third coronavirus episode in 2019 (Kong et al., [8]). Compared to the World Health Organization's (WHO) declaration of a pandemic, COVID-19 has started spreading and has affected at least 20 million people, with a mortality toll of around 5 lakh at the time of such an assessment (Worldometer, [9]). Due to the inadequacy of lab-based high throughput screening (HTS), virtual screening (VS) has emerged as a preferred tool for discovering effective molecules while hospitals continue to trial and error strategies for COVID-19 drug discovery (Jin et al., [10]; Kandeel and Al-Nazawi, [11]). The strategy of specifically targeting biomolecule (e.g., DNA, protein, RNA, and lipid) by using a computer program, of a cell to suppress its growth and/or activity is known as rational drug discovery which can be recognized by VS (Shoichet, [12]; Lionta et al., [13]). Two significant subgroups of this form of screening are ligand-based and structure-based drug design and discovery (Lionta et al., [13]; Yu and Mackerell, [14]; KeshavarziArshadi et al., [15]). Computationally and experiment-based access can determine the viral protein structures and antiviral candidates can be identified quickly and at a low cost using VS (Senior et al., [16]; Zhang et al., [17]). Furthermore, traditional vaccine development approaches are expensive, and developing an effective vaccine against a specific virus might take many years. COVID-19 vaccines have been developed and manufactured with much effort, and the efforts to advance vaccine clinical trials have been tremendous. Coronaviruses are positively stranded RNA

viruses that have their genome packaged into the nucleocapsid (N) protein and are surrounded by the membrane (M), envelope (E), and spike (S) proteins (Li, [18]). While many coronavirus vaccine experiments targeting various structural proteins were done, most of these efforts came to an end soon after the SARS and MERS outbreaks. The first human trial of the mRNA-based vaccination targeting the SARS-CoV-2's S protein began on March 16, 2020, as an expedient response to the COVID-19 pandemic. S protein is the coronavirus's most superficial and protrusive protein, and it plays a critical function in the entry of the virus. Because of their capacity to induce neutralizing antibodies that block host cell entry and infection, the full-length S protein and its subunit S1 (which contains the receptor-binding domain) have frequently been employed as vaccine antigens in the development of SARS and MERS vaccines. Current coronavirus vaccines, particularly S protein-based vaccines, may, however, have challenges with producing complete protection and potential safety concerns (Roper and Rehm, [19]; De Wit et al., [20]).

Nonetheless, sterile immunity and full protection are desired outcomes of a COVID-19 vaccine. Furthermore, it is becoming increasingly obvious that diverse immune responses, such as those elicited by cell-mediated or humoral immunity, are more important predictors of protection than antibody titers alone (Ong et al., [21]). One of the technology reverse vaccinology (RV), which is aimed at uncovering viable vaccine candidates through bioinformatics analysis of the pathogen genome, has transformed vaccine research in recent years.

2. Reverse Vaccinology

Reverse vaccinology (RV) was introduced in the early 1990s as a genome-based vaccine design approach (Rappuoli, [22]; Bullock et al., [23]) and attributed to the reason that bacterial culturing was no longer needed for selecting vaccine targets; the field was transformed to a more efficient status (Soria-Guerra et al., [24]; Heinson et al., [25]; Bruno et al., [26]). Its goal is to use bioinformatics to analyze the pathogen genome to find a good vaccination candidate. RV has been used to develop vaccines against infections like Group B meningococcus, which resulted in the approval of the Bexsero vaccine (Folaranmi et al., [27]). The research and application of vaccine-like compounds to humans date back to prehistoric times. Hilleman et al. drew a simple diagram to show this history (Figure 1). We are living in the present era of vaccine development, which is more effective and productive than any previous period in history, according to this diagrammatic overview. This advancement has been achieved due to generous financial support as shown in Figure 1 (Hilleman et al., [1]).

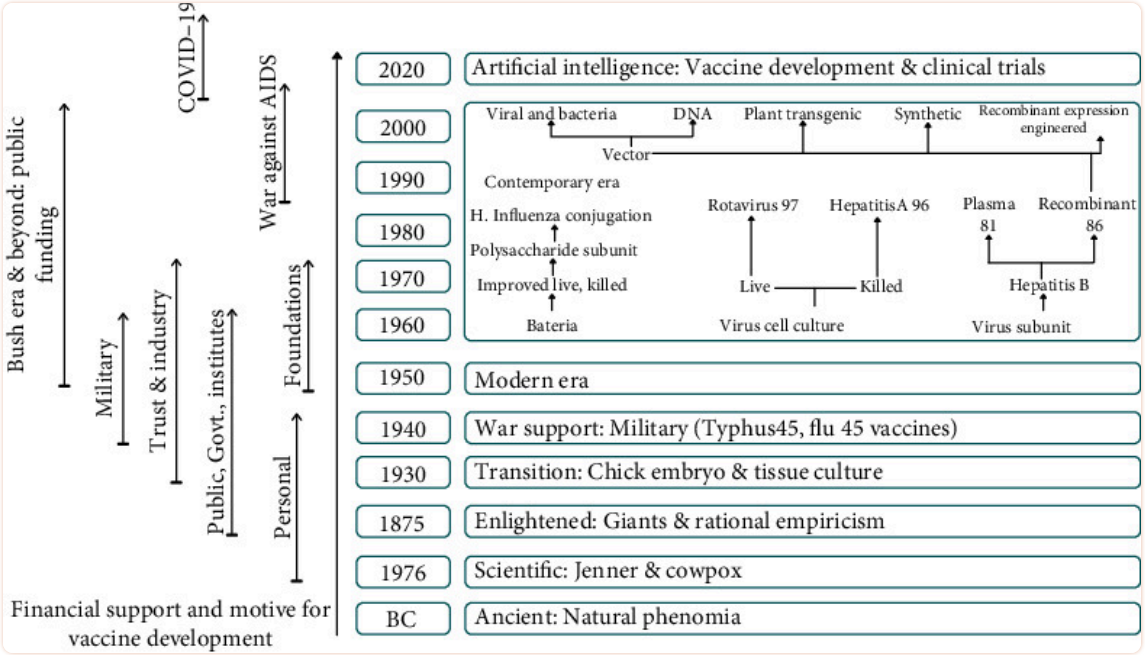

Figure 1

History and evolution of vaccine development by Hilleman.

In recent decades and for future perception, AI is the most emerging and demanding scientifically engineered technique. Through this, the computational understanding of machines can be obtained by incorporating intelligent behavior to innovate intelligent and smart machines. AI consists of various pieces of techniques, tools, and algorithms such as neural networks, symbolic AI, deep learning, machine learning, and genetic algorithms. These tools are growing and showing impact in different fields like military, space, robotics, and health. AI term was given by John McCarthy while he was at a conference on this subject in 1956 (McCarthy, [28]; Turing, [29]; Russell et al., [30]). In recent years, the advanced featuring tool of AI is machine learning (ML) reorganized in different fields of engineering and science. Nowadays, it is largely adopted in our daily lives, but the ability to find out the conceptual abstract from the large volume of data and feature learning is the most powerful contribution of ML as a tool of AI (Lecun et al., [31]; Grover and Toghi, [32]; Sun et al., [33]). The subbranches of AI are depicted in Figure 2.

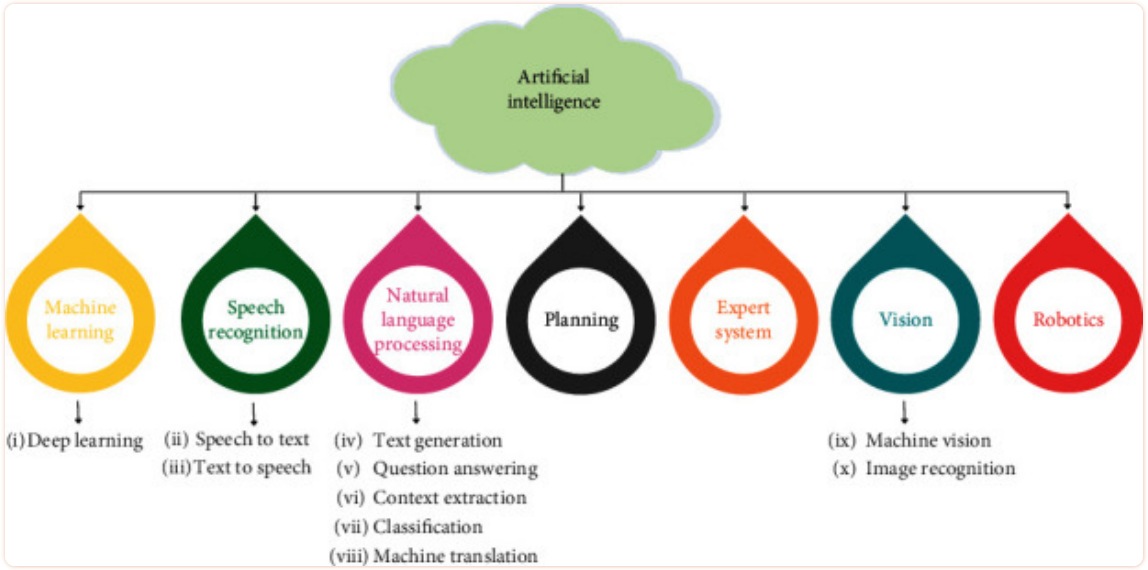

Figure 2

Subbranches of artificial intelligence.

AI is applied to medicine and has two main divisions, which are virtual and physical; the virtual part is acted by ML (also called deep learning), and its representation is achieved by mathematical algorithms, as a count of its experience; it improves learning. Based on ML algorithms, there are three divisions:

1. Supervised (prediction and classification algorithms based on former examples)
2. Unsupervised (patterns finding ability)
3. Reinforcement learning (rewards sequences and punishments are used to build a scheme for operation in a particular problem)

Earlier, AI has raised and is still raising the impact of its techniques in genetics and molecular medicine discoveries due to algorithms of machine learning and management of knowledge. A great example of success in the development of medicine and vaccine is determined by unsupervised protein-protein interaction algorithms, which can lead to remedial target discoveries (Theofilatos et al., [34]). Adaptive evolutionary algorithms and state-of-the-art clustering are the two methods used in the combination and that novel methodology is named "evolutionary enhanced Markov clustering." More than 5000 protein complexes are under this permitted pre-

diction from which at least one gene ontology function phrase reinforced over 70% of the results. The development of a novel computational methodology is permitted to identify single-nucleotide polymorphisms (SNPs) of DNA variants to predict the traits or diseases by employing revolutionary evolutionary. This works by embedding algorithms that are more robust and less prone to over-fitting problems that occur when a model has too many parameters with the number of observations (Rapakouliaet al., [35]; Theofilatos et al., [34]). According to the predictions, the most effective method for drug development is the Graph Convolution Neural Network (GCNN) (Duvenaud et al., [36]; Kearnes et al., [37]). These networks can retain graphs and extract properties from the information encoded within the compound characteristics. AI contributes to learning which can be successfully done by GCNN for a molecule and compound's prediction such as property and protein interface estimation (Fout et al., [38]; Liu et al., [39]). Some models are sequence-based like proteomics, transcriptomics, and genomics. They have shown an impact in recent years because of natural language processing (NLP) domain advancement, but more advanced generation models are context-based models which gain the attention from sequence-based models (Devlin et al., [40]).

Furthermore, vaccine development techniques are being adapted to certain countries' economic and health needs. This tendency has a direct impact on the goods in development as well as the quantity and types of clinical studies conducted. This chapter briefly summarizes the role of AI-based models in COVID-19 drug discovery or research and vaccine development (clinical trials). It is to be proposed that a concentrated effort be made to use AI methodologies to utilize information from preexisting data.

2.1. Coronavirus Structure

Coronavirus's basic structure is made up of five proteins, which are named as follows: spike proteins (S), Membrane proteins (M), envelope glycoproteins (E), nucleocapsid proteins (N), and hemagglutinin esterase (HE). Spikes are thought to have three heads (S1) and a trimeric stalk, according to several studies (S2). S1 connects to a host cell's particular surface receptors for viral attachment, whereas S2 binds to the viral and host membranes at the time of virus penetration. This makes it possible for the virus to infect the cells of the host (Li, [18]). The structural proteins are M proteins that aid in the determination of a virus's shape (Neuman et al., [41]). These are the most common proteins in CoVs. These proteins are primarily responsible for RNA packing (Tang et al., [42]). The protein E is extensively produced inside the infected cell during the replication cycle (Venkatagopalan et al., [43]). Furthermore, E protein is involved in viral budding, assembly, and morphogenesis (Nieto-Torres et al., [44]). Phosphoproteins with the ability to attach to the RNA genome are known as N proteins (de Haan and Rottier, [45]). The N protein is required for virion assembly, replication, and CoVs transcription. It aids in the budding and assembly of viruses (Tooze et al., [46]). Certain enveloped viruses have a glycoprotein called HE. The host cell surface receptor is a sialic acid derivative, and HE aids in its adhesion or recognition. It is the one who is responsible for the receptor's demise (de Groot, [47]; Zeng et al., [48]).

2.2. COVID-19: Molecular Mechanisms and Target Selection

The spikes on the coronavirus bind to a receptor present on the surface of a cell. After fusing with the cell membrane, the virus releases its RNA genome into the cell. The cell then produces copies of RNA as well as structural proteins required for the assembly of new virus particles, which are then discharged into the body. The role of the immune system is to destroy pathogens such as bacteria and viruses that are causing disease. Its first step is an innate immune response that is to send a variety of weapons to fight infection. But if the pathogens are new to the body and this response does not work to fight infection then, an adaptive immune response comes into action. During the adaptive immune response, specialized cells envelop the virus and deliver antigens, which activate immune cells. The two types of white blood cells are B cells and T cells which play a role in adaptive immunity. Antibodies are specialized proteins that are produced by B cells that bind to pathogens and prevent them from infecting healthy cells. T cells can destroy virus-infected cells that prevent them from replicating the virus. Meanwhile, memory B and T cells record the antigens, ensuring that the body responds rapidly if the coronavirus encounters again (Waltz, [49]). B cells are important in humoral immunity because they can secrete antibodies that neutralize the antigen. The ElliPro web tool (http://tools.iedb.org/ellipro/) was used to forecast both discontinuous and linear B cell epitopes. B cell epitopes assist in the detection of viral infections in the immune system. At the 0.51 threshold, ABCpred (http://crdd.osdd.net/raghava/abcpred/) was utilized to forecast 14-mer B cell epitopes for target proteins (Saha and Raghava, [50]; Ponomarenko et al., [51]; Rafi et al., [52]). T cell epitopes are important in the development of vaccines. It saves money and time as compared to laboratory experiments. 8–11 mer MHC class-I and 11–14 mer MHC class-II epitopes were predicted using the IEDB consensus technique (http://tools.iedb.org/mhcii/) (Zhang et al., [53]; Tahir ulQamar et al., [54]). Figure 3(a) shows a schematic representation of adaptive immunity targeting pathogens for killing.

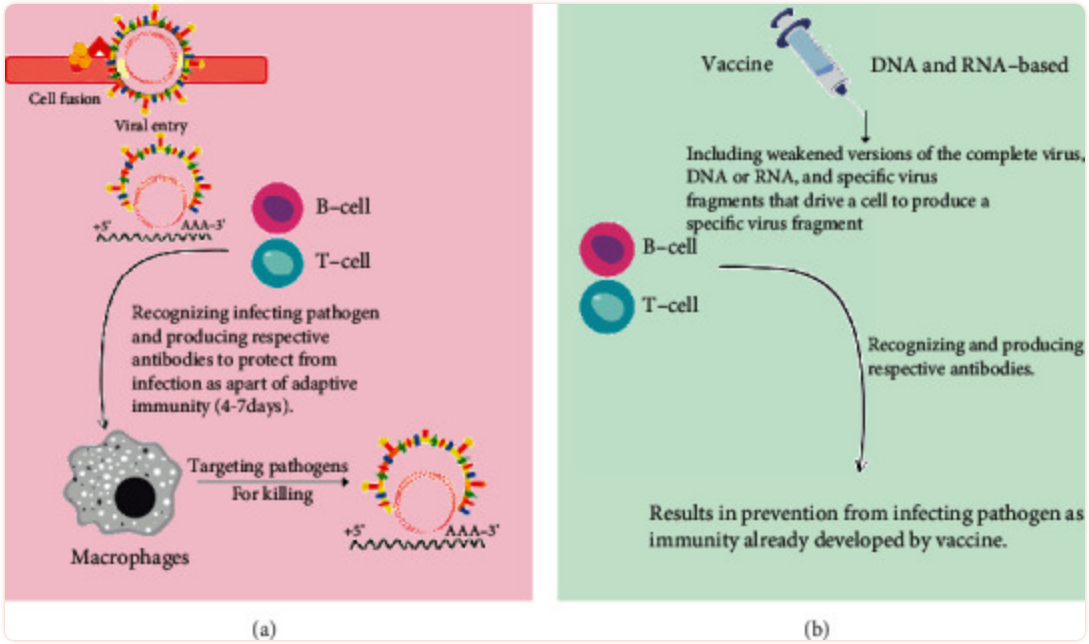

Figure 3

(a) Schematic representation of adaptive immunity targeting pathogen for killing. (b) Role of a vaccine in preventing the spread of a virus.

2.3. COVID-19: Vaccines

A vaccine usually contains an agent that looks like a disease-causing germ (microorganism) and is manufactured from weakened or destroyed microbes, one of their surface proteins, or their toxins. The agent induces the body's immune system to detect the agent as a threat as well as any microbes connected with it (Melief et al., [55]; Bol et al., [56]). Vaccines can be used as preventive or therapeutic measures (Brotherton, [57]; Frazer, [58]). Vaccines are the most significant public health measure against COVID-19 around the world as SARS-CoV-2 is a highly contagious virus infecting people all over the globe (Amanat and Krammer, [59]). Figure 3(b) shows the role of the vaccine in preventing the spread of a virus. COVID-19's spread shows no signs of halting, and its fatality rate is relatively high when compared to other viral-based infections; the development of vaccines and antiviral treatments against SARS-CoV-2 is very critical and essential. Most vaccines take years to develop ranging from 5 years for Ebola and 40 years for polio. On average, vaccines took 15 years. The vaccine trial process consists of various steps that must be followed quantitatively and systematically. The

length of this process is proportional to the vaccine's purpose and nature, which is to protect healthy people from pathogen infection (Deb et al., [60]; Thanh Le et al., [61]). As this virus is fatal, our societies and economies are unlikely to return to normal until a highly effective vaccination has been given to a large section of the world's population.

The hunt for a safe and effective vaccine was grown to be a massive project involving thousands of researchers working in laboratories pursuing different platforms for COVID-19 vaccine all over the globe. COVID-19 vaccine development platforms include several novel technologies. Vaccines work by presenting antigens that cause the immune system to respond without getting the individual sick. Vaccines come in a variety of forms, including weakened versions of the complete virus, DNA or RNA, and specific virus fragments that drive a cell to produce a specific virus fragment. The costly and long process of vaccine development can be accelerated using computational methods. However, because of the urgent necessity and deteriorating situation worldwide, some scientists (Pfizer) raced to develop COVID-19 vaccines. The pharmaceutical industry incorporated artificial intelligence in several areas of the development of vaccines and trials during the process. The scientists need to go through each data set for checking errors and other irregularities that occur naturally while collecting millions of data points. Advantageously, technology helped to reduce the effort smoothly. As reported by Cohen in Science magazine [62], the American company Moderna has reduced the time it takes to develop a human-testable vaccine prototype by utilizing bioinformatics technologies in which AI looks to play a crucial role. Moderna was one of the first to introduce a COVID-19 vaccination that was effective. Moderna is also utilizing artificial intelligence to aid in the development of mRNA sequences. Dave Johnson, an AI Officer, and Moderna's Chief Data show how robotic automation and AI algorithms allowed them to go from manually creating roughly 30 mRNAs per month to being able to manufacture over 1,000 per month. Its use of AI to speed up development was one of the reasons it was able to achieve this breakthrough so swiftly (Gast, [63]). AstraZeneca, a major contributor to the COVID-19 vaccine, is employing artificial intelligence not only in the development of the Covishield vaccine but also in drug discovery. AstraZeneca was one of the first companies to use artificial intelligence in the healthcare sector. To make the drug-making method less expensive, safer, and faster, the company has introduced AI into each step of the research and development process. AstraZeneca has combined knowledge graph and image analysis to get new insights into diseases and detect biomarkers 30% faster than human pathologists (Beatrice, [64]). Thermoregulated storage is required for the entirety of COVID-19 vaccinations. Covishield from Oxford-AstraZeneca and Covaxin from Bharat Biotech, for example, demand a storage temperature of 2–8°C. IoT based on sensor technology, which allows for continuous real-time data monitoring, can help to ensure a reliable storage system. If the temperature changes, the sensors will detect it and issue a device alert for the next vaccination shipment (Kumar and Veer, [65]). AI was successfully employed by Pfizer to run vaccine trials and expedite distribution. Pfizer, on the other hand, used AI throughout the vaccine development process to ensure that the COVID-19 vaccine met the needs of individuals. Pfizer began automating its research and development activities and incorporating artificial intelligence into its working system even before the epidemic. The company employed artificial intelligence algorithms to help identify signals amid millions of data points in its 44,000-person research during the vaccine trials. AI was applied in several aspects of vaccine development and trial during the vaccine development process by the pharmaceutical industry. After satisfying the key efficacy case counts, the data were analyzed and made available in approximately 22 hours with the help of an ML tool, i.e., Smart Data Query (SDQ). Throughout the study, the ML technique assured

data quality, requiring very little human interaction (Beatrice, [66]). Table 1 shows the implications of AI/ML in some of the vaccines for SARS-COV-2. Live attenuated, inactivated, and inactivated with adjuvant vaccines are still being developed using the traditional process. Recombinant subunit vaccines as well as more advanced approaches along with DNA and RNA-based vaccines are also being used (Thanh Le et al., [61]; Zhang et al., [17]; Lurie et al., [67]).

Table 1

Implications of AI/ML in some of the COVID-19 vaccines.

S. No.	Vaccines with manufacturer	Use of AI/ML	References
1.	AZD1222/Covishield AstraZeneca/Oxford University	Used graphical-based knowledge and image analysis to get new clues into illnesses and detect biomarkers 30 percent faster than human pathologists. ML techniques were recently used by AstraZeneca in pathology to speed up the assessment of tissue samples. With the use of big data analysis, AstraZeneca makes it much easier to mine electronic health records (EHR) to optimize clinical trial patient recognition and recruitment. ML and AI were already being used during clinical trials for event assessment to improve the process at various stages with the goal of lowering total duration. ML and AI were already being used during clinical trials for event assessment to improve the process at various stages with the goal of lowering total duration. IoT based on sensor technology came into use that allows for continuous real-time data monitoring and can help to ensure a reliable storage system. If the temperature changes, the sensors will detect it and issue a device alert for the next vaccination shipment.	Weatherall, [68], Kumar and Veer, [65], Sachdeva, [69]
2	mRNA-1273 Moderna	To aid in the designing of mRNA sequences, Moderna employs AI. They went from manually creating roughly 30 mRNAs (a molecule essential to the vaccination) per month to being able to generate almost 1,000 per month because of AI algorithms and robotic automation.	Gast, [63]

3. AI in Vaccine Research

Studying the proteins that make up the virus, such as the spike protein (S), is one of the roles and functions of AI in vaccine development. An AI system can sort through thousands of components in a complicated structure to find the ones most likely to elicit a strong immunological response. To ensure that a vaccine remains effective over time, AI systems must identify components that are unlikely to change or mutate. In the search for a vaccine, a crucial role has been seen for computational analyses and machine learning algorithms. These technologies are helping in assisting researchers in better understanding the virus with its structure and predicting which of its components will elicit an immune response, which is an important and main stage in vaccine development. These techniques can assist researchers in selecting the components for possibly potential vaccines and making sense of experimental data. By merging data from numerous experimental and real-world sources, AI allows scientists to derive insights. They also help in tracking the virus's genetic alteration (mutation) over time, which will determine the value of any vaccine in the future time (Waltz, [49]).

4. Different AI Tools

It has been documented that the National Institute of Allergy and Infectious Diseases funded the first clinical trial of an AI-based flu vaccine in 2019 in the United States (Ahuja et al., [71]). Flinders University scientists created the vaccine with the use of an AI tool called synthetic chemist, which generated trillions of synthetic chemical compounds. The researchers next used the Search Algorithm for Ligands (SAM), an AI program that sifts through trillions of molecules to determine which one might be a good vaccine adjuvant candidate (Park, [71]). This method can lessen the time it takes to develop a vaccine by several years. Screening compounds as potential adjuvants for the SARS-CoV-2 vaccine as well as a screening of new compounds based on modeling of probable changes or mutations to the novel coronavirus is easier with an AI-based approach. As the virus is having the potential to mutate, this will aid in the development of vaccines (Ahuja et al., [71]). It has never before been seen in human history for such a race to develop a vaccine against a virus. But by utilizing the power of AI, the velocity of discovery can be greatly enhanced. Figure 4 shows the process of discovering vaccine candidates via the AI/ML method.

Figure 4

Process of vaccine discovery by AI/ML method.

Over the last two decades, machine learning has also aided vaccine development. Machine learning-provided ligand-protein interaction, reaction prediction (Fooshee et al., [72]), activity prediction (Zhavoronkov et al., [73]), and compound property prediction (Ma et al., [74]) are the most affected domains of vaccine and drug discovery (Chen et al., [75]). RV is a technique for developing novel vaccines that begin with pathogen genome sequencing. Through bioinformatics analysis of the pathogen genome, RV tries to identify potential vaccine candidates. By selecting epitopes and screening vaccine candidates in silico, RV can be used to choose an antigen for a novel vaccine that can elicit an immunological response and also speed up and lower the cost of the process of vaccine development (Rappuoli et al., [22]; Mora et al., [76]; Hwang et al., [77]).

VaxiJen was the first application of machine learning in RV methods, and it has shown encouraging antigen prediction outcomes (Doytchinova and Flower, [78]; Heinson et al., [79]). Vaxign, the first web-based RV program (He et al., [80]), has been used to predict vaccine candidates against a variety of viral and bacterial infections (Xiang and He, [81]). Vaxign's first generation uses a filtering-based strategy to choose vaccine antigen candidates based on the user's past knowledge of the pathophysiology of the target pathogen (Ong et al., [21]). Recently, Vaxign-ML, a machine learning approach, has been developed to enhance prediction accuracy (Ong et al., [82]).Vaxign-ML used the biological and physicochemical properties of protein sequences as input variables to train five different machine learning models. The input protein sequences were taken from the Protegen database, which has been collecting and annotating experimentally confirmed protective antigens for the past ten years (Yang et al., [83]; Ong et al., [21]). In a study, data was gathered from the Immune Epitope Database (IEDB), the Virus Pathogen Resource, and the National Center for Biotechnical Information by a team from the University of Southern California. Over 600,000 epitomes from 3,600 distinct species are stored in the IEDB. When applied to SARS-CoV-2, the AI model immediately ruled out 95% of the elements that could be COVID therapies, highlighting the best alternatives. This AI tool predicted a total of 26 possible potential vaccines to combat the deadly infections. The researchers chose 11 of the 26 to use in the development of a multiepitope vaccine that targets the viral spike proteins that are important for replication. The suggested vaccine design framework can address the three most commonly observed mutations and may be

expanded to include other potentially unknown mutations. Several vaccines have been in use now, but in case the mutation occurs and possibly reduces the effectiveness of vaccines in use, the AI-assisted method will be able to provide quick results to design other preventive mechanisms. Currently, AI technology only employs B cell and T cell epitopes to generate findings. IgPred is a tool that predicts when immunoglobulin subclass a B cell epitope is capable of. The tool was trained using the support vector machines (SVM) method on over 14,000 epitopes and can be used to detect epitopes that induce IgG and IgA antibodies (Gupta et al., [84]; Khairkhah et al., [85]). NetCTLpan has been used in multiple SARS-CoV-2 vaccine development studies, and it provides end-to-end cytotoxic T cell epitope predictions (Mishra, [86]; Ayyagari et al., [87]). AI technology can create a stronger and faster vaccine if given more datasets and viable combinations. This approach is thought to be capable of accurately predicting over 700,000 distinct proteins (Komarraju, [88]). It is critical to forecast the peptides that bind multiple human leukocyte antigen (HLA) molecules to build and develop an effective vaccine for a large population (Brusic et al., [89]). MHC-I and MHC-II proteins encoded by the HLA gene present epitopes as antigenic determinants. Recursive feature elimination (RFE), SVM, and random forest (RF) are examples of machine learning algorithms that have been used to identify antigens from protein sequences (Bowick et al., [90]; Rahman et al., [91]). Detecting the presence of antigenic peptides presented by MHC-II is one of the most straightforward applications of ML and other AI-based technologies in vaccine development. The examples which can predict antigen presentation are MoDec and MARIA (major histocompatibility complex analysis with recurrent integrated architecture). To better understand natural immunity, various AI-related technologies have been employed to examine SARS-CoV-2 viral peptide presentation on MHC molecules from patients. Thus, it could aid either directly or indirectly in the discovery of COVID-19's unique immune response and the development of a successful vaccine. MARIA improves HLA-II prediction by integrating better training data with a novel supervised machine learning model that uses a multimodal recurrent neural network. (RNN). A similar technique to the convolutional neural network (CNN) is a motif deconvolution technique known as MoDec. It has been used to find out peptide cleavage and MHC-II binding motifs from MS-based peptidome datasets that compromises HLA-DP, HLA-DQ, and HLA-DR alleles. To detect B cell and T cell epitopes of SARS-CoV-2, Fast et al. [92] used two artificial neural network techniques known as MARIA and NetMHCPan4. The technique discovered 405 T cell epitopes with high MHC-I and MHC-II presentation scores, as well as two putative neutralizing B cell epitopes on the S protein. This discovery will aid in the development of effective COVID-19 vaccines and neutralizing antibodies (Racle et al., [93]; Chen et al., [94]; Moore et al., [95]; Arora et al., [96]). A recent study has used a combination of computational techniques and immunoinformatics, thus identifying antigenic epitopes in the structural proteins of SARS-CoV-2 (S, E, M) and suggested a plausible multiepitope-based subunit vaccine (MESV). MESV has sufficient structural and physicochemical characteristics to activate all components of the host immune system. It also seems to have a very stable interaction with the innate immune receptor toll-like receptor-3, making it more likely to enter the host immune system. By the use of computational tools, this study could help researchers save time and money when studying experimental epitope targets (Tahir ulQamar et al., [54]). The development of AI algorithms for determining whether a peptide binds numerous HLA molecules is extremely important in making the design of vaccines more time-efficient. The proposed systems include systems based on hidden Markov models (HMMs), artificial neural networks (ANNs) (Brusic et al., [97]), and SVMs (Bozic et al., [98]). SVM has been used to predict antigens in an RV problem (Heinson et al., [79]). For both MHC-I AND MHC-II binding peptides, RANKPEP provides a position-specific score matrix (Reche et al., [99]). MHCnuggets is a neural network model based on MHC

binders that have been trained on common and rare alleles (Shao et al., [100]). Lopez-Rincon et al. [101] proposed a CNN to classify 553 genome sequences with promising accuracy results for analyzing COVID-19 gene sequences. By giving a wide range of T cell epitopes, Monte Carlo-based simulation has been applied to forecast blueprinting for SARS-CoV-2 vaccines (Malone et al., [102]). It has been documented that the neural network method, NetMHC, predicts which peptides will bind and hence identify epitopes for the SARS-CoV-2 vaccine (Prachar et al., [103]). The application of AI in the research of vaccines and COVID-19 treatment is gaining a lot of attention due to international projects such as CoronaDB-AI, a data collection with genomic features that can be used to train AI models for COVID-19 treatments (KeshavarziArshadi et al., [104]; Liu et al., [105]). Figure 5 shows AI-based vaccine development for COVID-19. Recent studies have used hidden Markov models, Monte Carlo-based simulation, and neural network approaches to predict epitopes, the portion of an antigen that might stimulate an immune response, as a potential target in the development of a vaccine (Crooke et al., [106]; Prachar et al., [103]; Malone et al., [102]). A deep learning approach (DeepVacPred) has been used for predicting and designing a multiepitope vaccine that could predict 26 different SARS-CoV-2-spike-protein sequence vaccine components (Yang et al., [83]). Deep convolutional neural networks have proven to be a more reliable alternative for predicting MHC and peptide binding (Han and Kim, [107]). Deep learning autoencoders have shown promise in extracting features of human Leukocyte Antigen (HLA-A), which could be used in the development of a vaccine (Miyake et al., [108]). The network of long short-term memory has also shown some encouraging results. This type of RNN was used to predict epitopes for spikes (Abbasi et al., [109]). Malone et al. [102] used a similar strategy, employing deep learning RNN and simulated spike sequences to discover potential vaccination targets. RNN gave sequences for a protein of interest with a high degree of sequence identity. Malone et al. [102] used BepiPred, IEDB, and NEC Immune Profiler tools to create an epitope map for different HLA alleles and studied the complete SARS-CoV-2 proteome beyond spike, to give a comprehensive vaccine design blueprint for SARS-CoV-2. For the development of a vaccine for COVID-19, NLP models, notably language modeling techniques, have made a great impact. Pretrained transformers were utilized in carbohydrate chemistry to predict protein interaction (Nambiar et al., [110]) and model molecular processes (Abbasi et al., [109]), which can be applied in the vaccine development process. The transformers were employed to repurpose commercially available medications by anticipating their interactions with SARS-CoV-2 viral proteins (Beck et al., [111]). Researchers at the University of Basel in Switzerland utilized a protein-modeling tool called the Swiss Model to anticipate the architectures of proteins on the outer surface of the SARS-CoV-2 virus when the pandemic struck. DeepMind, a London-based AI firm, used its AlphaFold neural network to predict the three-dimensional form of SARS-CoV-2 proteins based on the virus's genomic code (Waltz, [49]). Their predictions turned out to be accurate when compared to the virus's actual protein structures. Table 2 shows some of the common tools for the prediction of epitopes and their evaluated accuracy or reported accuracy, if available.

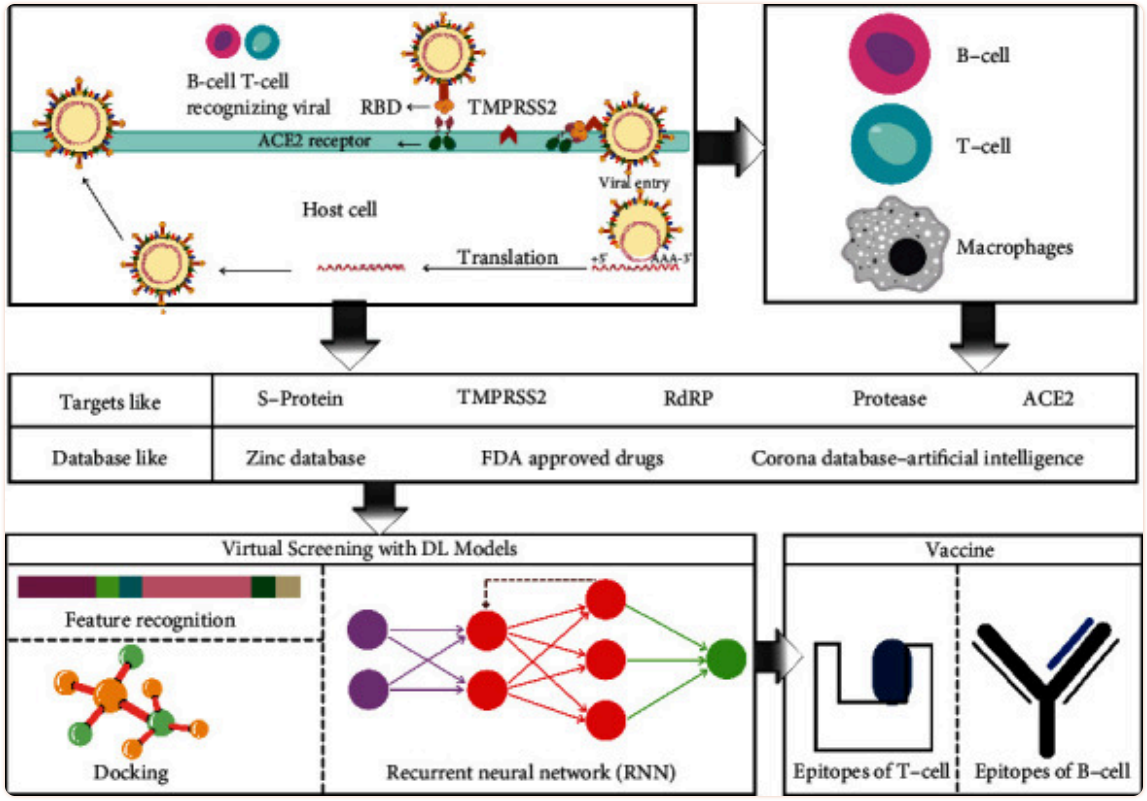

Figure 5

AI-based vaccine development for COVID-19.

Table 2

Some tools for the prediction of epitopes.

Technique	Method	References
RANKPEP	Prediction of MHC binding peptides, for MHC-I accuracy, is 80% and MHC-II is 0.96 AUC	He et al., [112]; Yazdani et al., [113]
MHCnuggets	A neural network (LSTM) model based on MHC binders that have been trained on common and rare alleles and self-reported accuracy of 0.924 AUC	Campbell et al., [114]
NetCTLpan1.1	Prediction tool for MHC-I epitopes and self-reported accuracy of 0.976 AUC	Ayyagari et al., [87]; Mishra et al., [86]; Safavi et al., [115]
BepiPred (2.0)	RF-based and ML-based models trained on epitopes and self-reported accuracy of 0.62 AUC	Rahman et al., [116]; Ayyagari et al., [87]; He et al., [112]; Khairkhah et al., [85]
DeepVacPred	Prediction and designing of a multi-epitope vaccine	Yang et al., [83]
IgPred	SVM-based B cell epitope prediction tool can be used to remove candidates with a high resemblance to IgE epitopes	Gupta et al., [84]

Therefore, the AI-powered technology offered a ray of hope by assisting scientists and medical health providers in dealing with this deadly disease. To summarize the role of AI in research, AI helps in gathering and synthesizing the information, determining the cause of the disease, and selecting and developing potential drug/vaccine candidates. Furthermore, after the selection of a potential candidate, AI helps in clinical trials of the vaccine.

5. AI in Clinical Trials of the Vaccine

The most time-consuming aspect of vaccine research and development is the testing of a vaccine. To acquire a better knowledge of the disease and for the research of a potential vaccine candidate and accelerate its speed, computational approaches and AI techniques have been developed as shown in Figure 6. Several applications of AI help in the rapid classification of novel viruses by detecting their intrinsic genomic structures and can be used to recognize similarities with other pathogens (Randhawa et al., [117]). Only one out of ten molecules get approved for entering into the clinical trials, which is a huge loss for the industry (Hay et al., [118]).

The reason for these failures can be caused by a lack of technical needs or infrastructure and poor patient selection. However, with a large amount of digital medical data available, AI can help in reducing these failures (Harrer et al., [119]). Moreover, different AI algorithms help in identifying the potential candidate for clinical trials of the vaccine in an efficient time after the screening of several candidates. Some researchers applied SDQ for reviewing the data of clinical trials in less than 24 hours for the same type of evaluation instead of more than 30 days. The rapid data access was accompanied by exceptional levels of good data quality resulting in a dependable outcome. As a result, the time between molecules to market was reduced from ten years to one year (Kulkarni et al., [120]). After the research for a novel vaccine candidate, it has to undergo a development process. The process is divided into preclinical and clinical trials by regulatory bodies all around the world (Singh and Mehta, [121]). The World Health Organization (WHO), the US Food and Drug Administration (USFDA), and the European Medicines Agency (EMA) have issued guidelines to plan the clinical development path of a potential vaccine candidate. Each vaccine develops in its way, based on the factors including the type of vaccine (peptide/DNA/RNA/inactivated/live), target population, and disease epidemiology. A vaccine candidate normally undergoes three phases of human development known as clinical trials, which are Phase I, Phase II, and Phase III trials before regulatory approval. To monitor the safety and efficacy in the population Phase IV trial is used after the completion of Phase III trials (Farrington and Miller, [122]; WHO technical report, [123]; Hudgens et al., [124]; Collins, [125]).

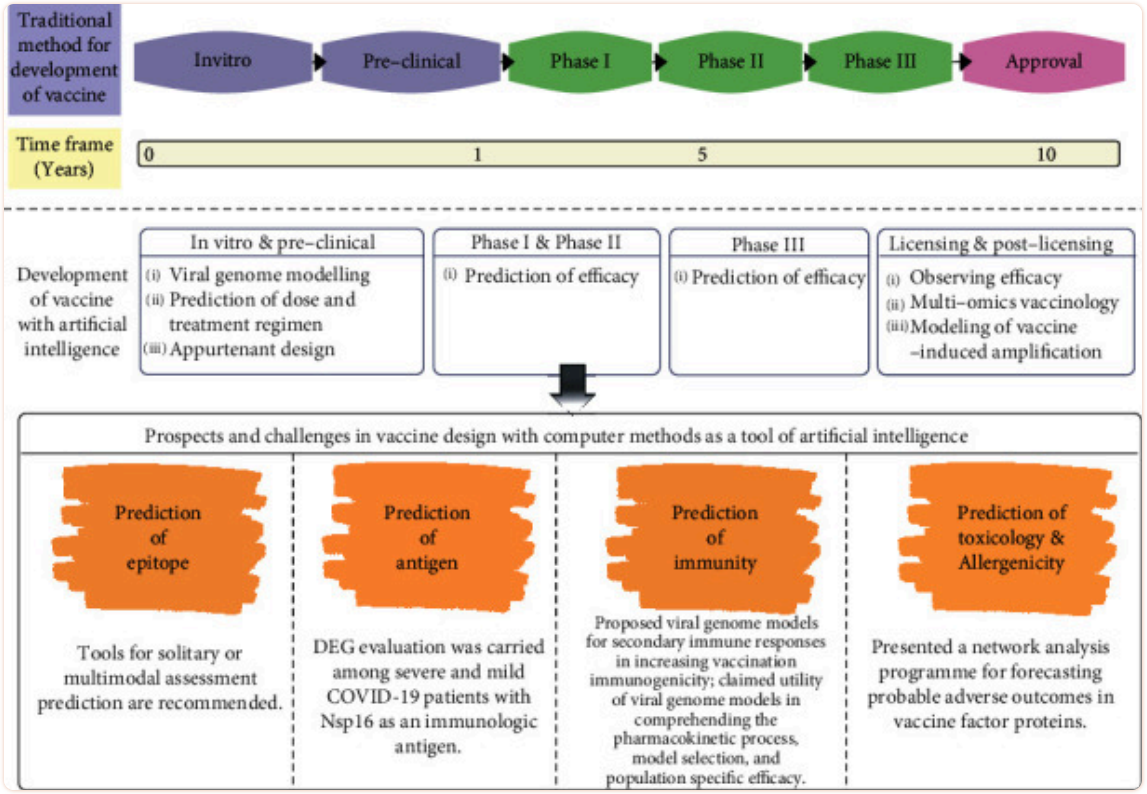

Figure 6

Benefits of employing computational methodologies in vaccine development. The bottom box outlines the points of view and issues raised at each stage of the proposed computational design tools. Processes connected with reverse vaccinology are shown in orange boxes at the bottom.

5.1. Different Phases during Clinical Trials of Vaccine

5.1.1. Phase I (20-80 Subjects) The phase I trial involves healthy subjects. It consists of the administration of the vaccine to subjects. The aim of this trial is the detection of safety and collection of an immune response. The immunization schedule, model of vaccine administered, and dose are often evaluated (Who technical report, [123]; Hudgens et al., [124]; EMEA, 2005).

5.1.2. Phase II (100-300 Subjects) After reaching a successful outcome in the phase I trial in terms of both immunogenicity and safety, a vaccine candidate should proceed to phase II clinical evaluation. The goal is to provide more information on safety, immunogenicity, and data on the optimal dose, vaccine preparation, and schedule of the vaccine to be taken up for the phase III confirmatory trial (Artaud et al., [126]).

5.1.3. Phase III (Large-Scale Population, 300-3000 Subjects) The effect of a final formulation is assessed in the phase III trial and is very important for the registration and approval of a vaccine to market. Safety and efficacy are the main goals of these trials. If the vaccine's efficacy and safety are demonstrated in the phase III trial, the manufacturer of the vaccine can submit an application to the national regulatory authority for a license and commercialize the product (Singh and Mehta, [121]).

5.1.4. Phase IV (Several Thousand People) It is also known as postmarketing surveillance studies (PMS). This trial is used to continue to monitor the vaccine for safety and effectiveness in the population. It is carried out after the successful completion of phase III trials and following licensure of the product (Singh and Mehta, [121]).

The various vaccine candidates are classified according to the technology used for the vaccine development. The list of a few vaccines with their characteristics, manufacturer name, phase 3 trial data, and efficacy data are presented in Table 3 (Kyriakidis et al., [127]).

Table 3

List of a few vaccines with their characteristics, phase 3 trial data, and efficacy data (Kyriakidis et al., [127]).

Vaccine type	Candidate vaccine name	Manufacturer(s)	Phase 3 trial starting date	Number of participants	Antibody response rate	Clinical trial registration number	Efficacy	Bene featu
Replication-defective viral vector vaccine	(1) Ad5-nCoV (2) AZD1222 (3) Sputnik V/Gam-COVID-Vac (4) JNJ-78436735/Ad26.COV2.S	(1) CanSino Biological/Beijing Institute of Biotechnology/Academy of Military Medical Sciences (2) AstraZeneca/Oxford University (3) Gamaleya Research Institute/Health Ministry of Russian Federation/Acellena Contract Drug Research and Development (4) Janssen Pharmaceutical Companies of Johnson & Johnson/Beth Israel Deaconess Medical Center	(1) September, 2020 (2) August, 2020 (3) September 7, 2020 (4) September 23, 2020	(1) 40,000 (2) 30,000 (3) 40,000 (4) 90,000	(1) Neutralizing antibodies produced in 97% of the participants (Phase 2) (2) Neutralizing antibodies produced in all participants that received the prime-boost regime (Phase 1/2) (3) Neutralizing antibodies produced in all participate (Phase 1/2) (4)	(1) NCT04526990 (2) NCT04516746 (3) NCT04530396 (4) NCT04614948	(1) N/A (2) 62.1% overall and 90% (18 to 55 years old) (3) 91.4% (4) N/A	Can i hu cellu with Good

The majority of the work turns to testing once a vaccine candidate has been designed and developed. Vaccines are initially evaluated in the lab on cells and animals known as preclinical trials, then on human beings known as clinical trials. Unfortunately, AI tools cannot take the place of those time-consuming procedures. However, by applying patient-specific genome-exposome profile analysis, AI can help choose only a certain diseased population for engagement in Phase II and III clinical trials, allowing for early prediction of the viable therapeutic targets in the chosen patients (Mak and Pichika, [128]; Harrer et al., [119]). Predicting lead compounds and preclinical discovery of molecules before the start of clinical trials using AI tools such as predictive ML aid in the early prediction of lead molecules that will pass clinical trials with a selected population of the patient (Harrer et al., [119]). AiCure helps in monitoring the regular medication intake by patients with schizophrenia in phase II trials, resulting in a 25% increase in patient adherence and ensuring the clinical trial's success (Mak and Pichika, [128]). NLP can help in extracting and analyzing important data from patients' EHR records, can compare it to eligibility criteria for ongoing trials, and suggests matching studies. AI tools might be able to forecast which antigens the immune system will encounter, but what the immune system will do in a live human is beyond today's computer capabilities. Because the human body is so complex, AI tools cannot predict what the vaccine candidate will do for the body with reliable data. However, there was no evidence found that clinical trials were conducted using computational supervision. Although AI cannot anticipate the outcome of clinical trials, it can make sense of the mountains of data generated by these trials by analyzing all the factors and identifying patterns that a human brain might miss. As thousands of patients will be engaged as the vaccine candidate progress to the second and third phases of clinical testing, AI tools or systems will be very much critical in quickly assessing clinical and immunological data (Waltz, [49]; Piccialli et al., [129]). To summarize the role of AI in clinical development, it helps in trial planning, optimizing the recruitment process, risk monitoring, toxicity prediction, and also monitoring of drug adherence.

6. Conclusion

This paper summarized the application of AI/ML in the field of research and development of the SARS-COV-2 vaccines. AI has been shown as an emerging and promising technology for detecting early coronavirus infection and monitoring the state of affected individuals. AI techniques including ML and DL have shown to be beneficial to aid in the study of the virus by examining the data available and assisting in the development of proper treatment regimens as well as in the development of a novel vaccine candidate. The AI algorithms have become more important for advanced analysis and translation of basic discoveries into novel vaccine candidates due to the large accessible amount of data. However, AI approaches cannot replace the time taking tasks such as laboratory experimentations and clinical trials, but they can aid in the planning of a trial, monitoring, and predictions of deleterious and risk factors. This paper suggested the various computational tools and techniques developed based on AI and ML which can anticipate complicated immune system activities, such as B cell and T cell epitope prediction. With the help of computer-based tools and algorithms, it considerably improves decision-making, and treatment uniformity, and resulted in the speedup in vaccine development and research to fight against the SARS-COV-2 virus.

Data Availability

No data were used.

Conflicts of Interest

The authors declare no conflict of interest.

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REVIEW

Potential Effect of DPP-4 Inhibitors Towards Hepatic Diseases and Associated Glucose Intolerance

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Abstract: Dipeptidyl-peptidase-4 (DPP-4) is an enzyme having various properties and physiological roles in lipid accumulation, resistance to anticancer agents, and immune stimulation. DPP-4 includes membrane-bound peptidases and is a kind of enzyme that cleaves alanine or proline-containing peptides such as incretins, chemokines, and appetite-suppressing hormones (neuropeptide) at their N-terminal dipeptides. DPP-4 plays a role in the final breakdown of peptides produced by other endo and exo-peptidases from nutritious proteins and their absorption in these tissues. DPP-4 enzyme activity has different modes of action on glucose metabolism, hunger regulation, gastrointestinal motility, immune system function, inflammation, and pain regulation. According to the literature survey, as DPP-4 levels increase in individuals with liver conditions, up-regulation of hepatic DPP-4 expression is likely to be the cause of glucose intolerance or insulin resistance. This review majorly focuses on the cleavage of alanine or proline-containing peptides such as incretins by the DPP-4 and its resulting conditions like glucose intolerance and cause of DPP-4 level elevation due to some liver conditions. Thus, we have discussed the various effects of DPP-4 on the liver diseases like hepatitis C, non-alcoholic fatty liver, hepatic regeneration and stem cell, hepatocellular carcinoma, and the impact of elevated DPP-4 levels in association with liver diseases as a cause of glucose intolerance and their treatment drug of choices. In addition, the effect of DPP-4 inhibitors on obesity and their negative aspects are also discussed in brief.

Keywords: DPP-4, insulin, incretins, glucose intolerance, liver diseases, sitagliptin, DPP-4 inhibitors

Introduction to DPP-4 Enzyme

In 1966, Hopsu-Havu and Glenner found dipeptidyl peptidase-4 (DPP-4) in rat liver during the processing of the cells and commercially enzymatic preparations as an activity that liberates naphthylamine from Gly–Pro-2-naphthylamide, and it was originally called glycylproline naphthylamidase.[1] Meanwhile, the protein characteristics and distribution were intensively investigated, and it was rediscovered numerous times as a binding protein and a cellular marker.[2] DPP-4 is the enzyme for the immune response which is known as antigen CD26 co-stimulator of T- cell, having a multiuse protein that serves as a binding protein and a ligand for a range of extracellular molecules in addition to its catalytic activity.[3] It is a membrane protein that is expressed on cells all over the body, but it is also detached from the membrane and comes into circulation in the plasma as a soluble protein.[4,5] Lymphocytes, fibroblasts, endothelial cells, and apical portions of acinar and epithelial cells express DPP-4, which is also found in plasma as in soluble circulating form.[6,7]

All membrane-bound molecules like proline or alanine-specific exopeptidases have been proposed to have a biological function in the degradation of bioactive peptides,[8] but the DPP-4 role has been explored and reported most. In comparison to other peptidase enzymes, like aminopeptidase and carboxypeptidase, which have a limited distribution, DPP-4 is found in almost all vertebrate tissues, but its activity varies greatly.[9]

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The enzyme is found largely in the cortical region and in the brush-border and microvillus portions of the kidney and hepatocytes at the cytoplasmic membrane surrounding bile canaliculi and on epithelial of the bile duct in the liver. It can also be detected on pancreatic duct epithelial cells.[10] DPP-4 is thus present in body compartments/fluids engaged in nutrition and excretion (bile, pancreatic fluid, intestinal lumen, urine). As a result, DPP-4 plays a digestive role in the final breakdown of peptides produced by other endo and exo-peptidases from nutritious proteins and their absorption in these tissues.[11] In both rats and humans, DPP-4 is a ubiquitous enzyme, including the exocrine pancreas, biliary tract, spleen, small intestine, and brain.[12,13] DPP-4 possesses differentially expressed biological functions, as evidenced by its extensive organ distribution. The liver is among the organs with the highest levels of DPP-4 expression.[14] DPP-4 marking is high in hepatic acinar zones 2 and 3, but never in zone 1, in a normal healthy liver.[15] DPP-4 may be implicated in the control of hepatic metabolism, based on the uneven lobular distribution.[16]

DPP-4, on the other hand, is in direct touch with hormones flowing in the blood, as it is present on blood vessels' endothelial cells[17] and as a mobile enzyme in plasma. DPP-4 is expressed on excited T-helper lymphocytes[18] as well as fractions of macrophages[19] among immune system cells.[20] DPP-4 is highly expressed in the endocrine organs, but occasionally in parenchymal cells, such as thyroid follicular epithelial cells and luteal cells.[21] DPP-4 is expressed in specialized fibroblasts in a variety of tissues, including the skin, mammary gland, and synovia.[22] The concentration and activity of DPP-4 in different organs/tissues/cells are shown in Figure 1.

Molecular Biology of DPP-4

DPP-4 includes membrane-bound peptidases like fibroblast activation protein (FAP)/seprase, resident cytoplasmic enzymes, and nonenzymatic members, which are found in neuronal membranes, as well as prolyl endopeptidase. Despite other major changes in sequence, the position and identity of the residues are crucial for catalytic activity within the C-terminal region of these related enzymes and are highly conserved in prokaryotes and eukaryotes.[23] DPP-4 interacts with other membrane proteins and sends signals across cell membranes. The molecular structure of DPP-4 is shown in Figure 2.

Notably, the majority of the protein is extracellular, including the catalytic domain at the C-terminus, a cysteine-rich region, and a large glycosylated region connected to the transmembrane portion by a flexible stalk. Only six amino acids at the N-terminus are expected to reach into the cytoplasm. DPP-4 can form tetramers between two soluble proteins or

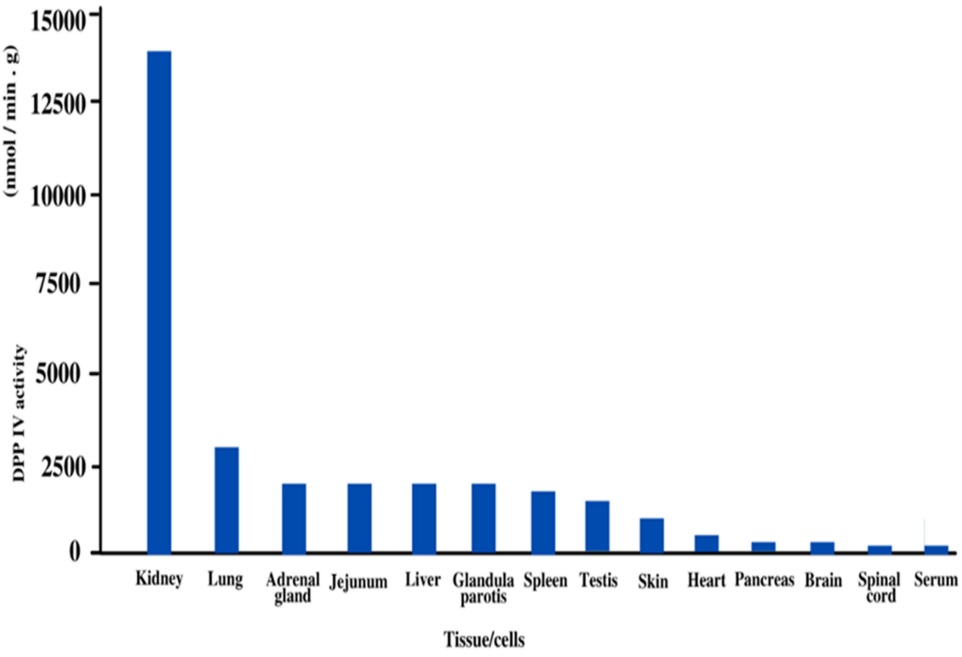

Figure 1 Graphical representation of the concentration and activity of DPP-4 in different organs/tissues/cells.

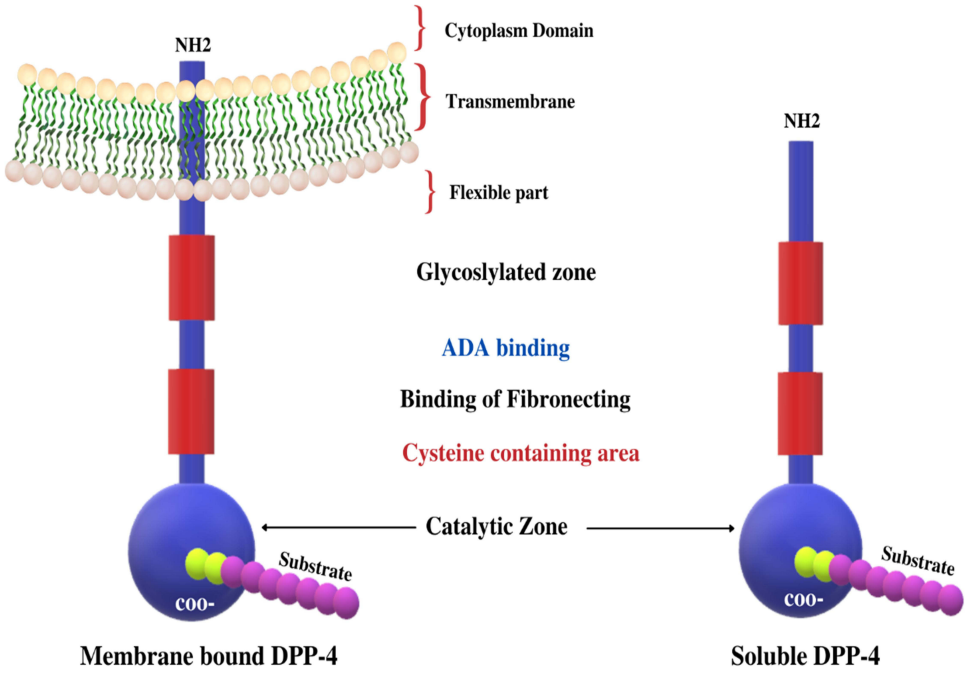

Figure 2 Molecular structure of DPP-4.

two membrane-bound proteins, which could alter the efficiency of substrate entrance and cleavage by the catalytic active site or facilitate cell–cell communication, as reported in a study of the protein crystal structure.[23]

The intracellular signalling of membrane-bound DPP-4 is initiated by the interactions with T-cell antigen CD-45, Adenosine deaminase (ADA), caveolin-1, and the caspase recruitment domain-containing protein 11.[24,25] DPP-4 binds to the extracellular matrix proteins, collagen, and fibronectin, as well as ADA, binding to these proteins and ADA, is mediated by amino acid residues that are not part of the substrate-binding site[26,27] (Figure 2). DPP-4 which is catalytically active is released from the plasma membrane, resulting in DPP-4 (727 aa), a soluble circulating form that lacks the intracellular tail and transmembrane portions (cytoplasmic domain, flexible stalk)[28,29] and accounts for a significant amount of DPP-4 activity in human blood.[30] Moreover, both membrane-bound and circulating soluble DPP-4 share some domains such as ADA binding domain, glycosylated region, cytosine-rich domain, catalytic domain, fibronectin domain, and the disulfide bonds.[25] Here are some examples of target peptides of DPP-4 as shown in Table 1.

DPP-4 Physiological Properties

DPP-4 is a kind of enzyme that cleaves alanine or proline-containing peptides such as incretin, chemokines, and appetite-suppressing hormones (neuropeptide) at their N-terminal dipeptides. GLP-1, peptide YY, GLP-2, chemokine ligand 12/stromal-derived factor-1 (CXCL12/SDF-1), and substance P are examples of potential targets. Consequently, DPP-4 peptidase activity has different modes of action on glucose metabolism, hunger regulation, gastrointestinal motility, immune system function, inflammation, and pain regulation. Figure 3 shows that DPP-4 has different modes of action on chemokine production and metabolism through its peptidase activity. DPP-4 is also implicated in immunological stimulation, anti-cancer drug resistance, and ECM (Extracellular Matrix) binding and breakdown. DPP-4 also has an impact on lipid build-up.

Role of Incretins and DPP-4 in Glucose Regulation

The functions and abundance of DPP-4 in the body have already been discussed in the above section. But the major focus is on the cleavage of alanine or proline-containing peptides such as incretins by the DPP-4 and its resulting consequences.

Table 1 Various Target Peptide of DPP-4

Peptide	Function	Reference
GIP Glucagon PACAP-38 GLP-1	Glucose metabolism	[31–34]
Peptide YY	Appetite regulation	[35]
IGF-1 GHRH GLP-2 VIP NPY GRP	Growth Gut Motility	[36,37] [35,38–40]
CCL11/eotaxin CXCL9/Mlg CCL22/MDC CCL5/RANTES CXCL10/IP10 CXL12/SDF-1 CXCL11/I-TAC	Chemokine	[41–47]
Prolactin hCGα LHα	Reproduction	[36,37,48]
Enkephalin Endomorphins Substance P	Pain regulation	[49–51]
Thyotrophin α	Homeostasis	[52]
Vasostatin-1	Endothelial cell growth inhibition	[53]

Incretins are hormones with an important role in the homeostasis of glucose, type 2 diabetes pathophysiology, and other metabolic disorders.[54] These incretin hormones help in lowering the blood glucose level by stimulating the release of insulin and insulin opens the GLUT4 channel so that glucose can enter the cell and is utilized by the cells for energy production.[55] There is an interesting fact that oral administration of glucose stimulates more insulin release than the intravenous administration of glucose while the concentration of glucose reaches circulation remains the same.[56] This situation is known as the incretin effect and it is credited to specialized cells enteroendocrine present in the gut and coupled with glucose absorption. When glucose is administered orally, it reaches the enteroendocrine cells during absorption, and incretin hormones like glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide (GLP-1) are released from enteroendocrine cells, which stimulate pancreatic β-cells to release insulin.[57] On the other hand, in the intravenous administration of glucose, the enteroendocrine cells are bypassed and thus less availability of incretins leads to less stimulation of pancreatic β-cells as compared to oral administration of glucose at the same concentration.[56,58] When blood glucose concentrations rise beyond a threshold of roughly 66 mg dL^{-1}, gut hormones including incretins generated in response to dietary absorption of glucose which provides the endocrine signal to the pancreatic β-cells, boosting insulin production and modifying glucagon secretion.[59] Incretin hormones stimulate insulin secretion physiologically, whereas physiological degrees of hyperglycemia constitute to provide a stimulus accordingly for the release of insulin.[56,60,61] An "isoglycemic" intravenous glucose administration induces an identical increase in arterial blood glucose level just as an oral glucose load leads to a rise in insulin secretion that is around one-third of the

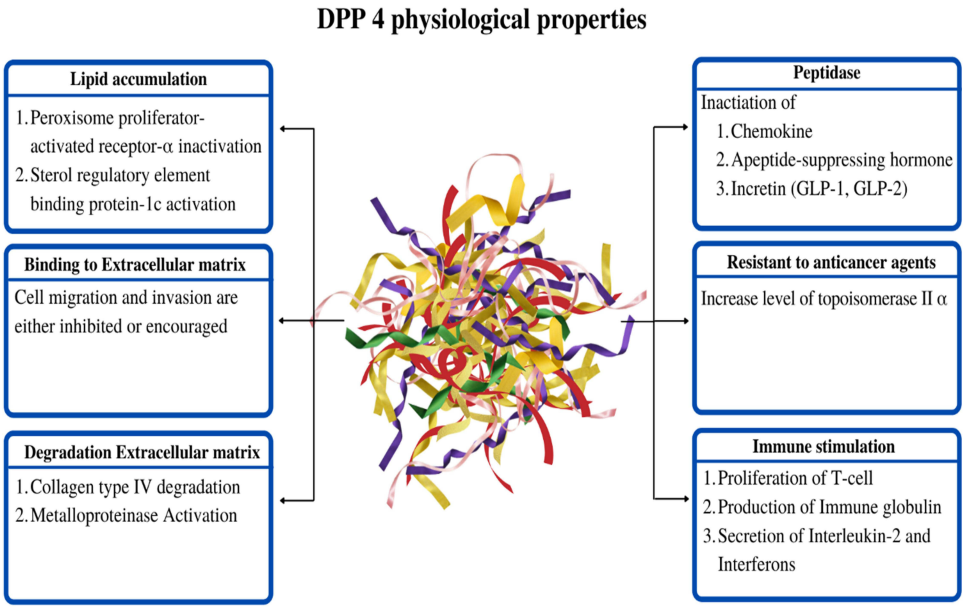

Figure 3 Physiological properties of DPP-4 in various regions.

stimulation responses induced by oral glucose, which is the combined action of hyperglycemia and incretin hormones.[62] The contribution of incretin hormones in the secretion of insulin responses following oral glucose administration is estimated to be in the range of 25% and 75%, depending on the dosage of glucose used. Undoubtedly, this measurable contribution supports incretin hormones' physiological role in the maintenance of normal glucose homeostasis.[56] The endocrine pancreas receives three signals from the gut, which is possible due to three substrates viz. incretin hormones, glucose, and neural signals by the autonomic nervous system.[62,63]

After the utilization of glucose by the cells throughout the body, insulin release is reduced accordingly and extra available incretins are degraded by the enzyme DPP-4 as a part of homeostasis. However, excess availability of enzyme DPP-4 leads to a condition by unnecessarily inhibiting the activity of incretins, which leads to a reduction in the secretion of insulin, and reduced insulin is not able to open the sufficient amount of glucose channels GLUT4 leads to cause glucose intolerance or hyperglycemia. As the intestinal hormone, glucagon-like peptide-1 (GLP-1) was discovered to be a DPP-4 substrate, the relationship between DPP-4 and glucose homeostasis was discovered.[64,65] GLP-1 role in managing glycemia was discovered in 1986[66] when this unknown peptide was discovered to have dramatic effects on the endocrine pancreas. Denmark and the United States researchers described potent insulinotropic[67] and glucagonostatic effects.[68] Whenever the level of glucose increases then incretins stimulate the release of insulin which lowers the blood glucose, but when the DPP-4 level increases due to any cause, it metabolizes the GLP-1 and reduces the availability of the incretin hormones. The level of glucose continuously increases but incretin hormones are unable to stimulate insulin release which can result in hyperglycemia or glucose intolerance due to the high availability of DPP-4[32] (Figure 4). It is observed that the level of DPP-4 is increased in various liver conditions. The pathological role of DPP-4 in liver diseases and associated glucose intolerance with their therapeutic management are discussed below in detail.

DPP-4 in Liver Conditions and the Potential Effect of DPP-4 Inhibitors in Reducing the Risk of Liver Conditions

As per research, as the DPP-4 level increases in individuals with liver conditions[69–71] and up-regulation of hepatic DPP-4 expression is likely to be the cause of glucose intolerance or insulin resistance.[72,73] The effects of DPP-4 on each liver disease with pathology are described below.

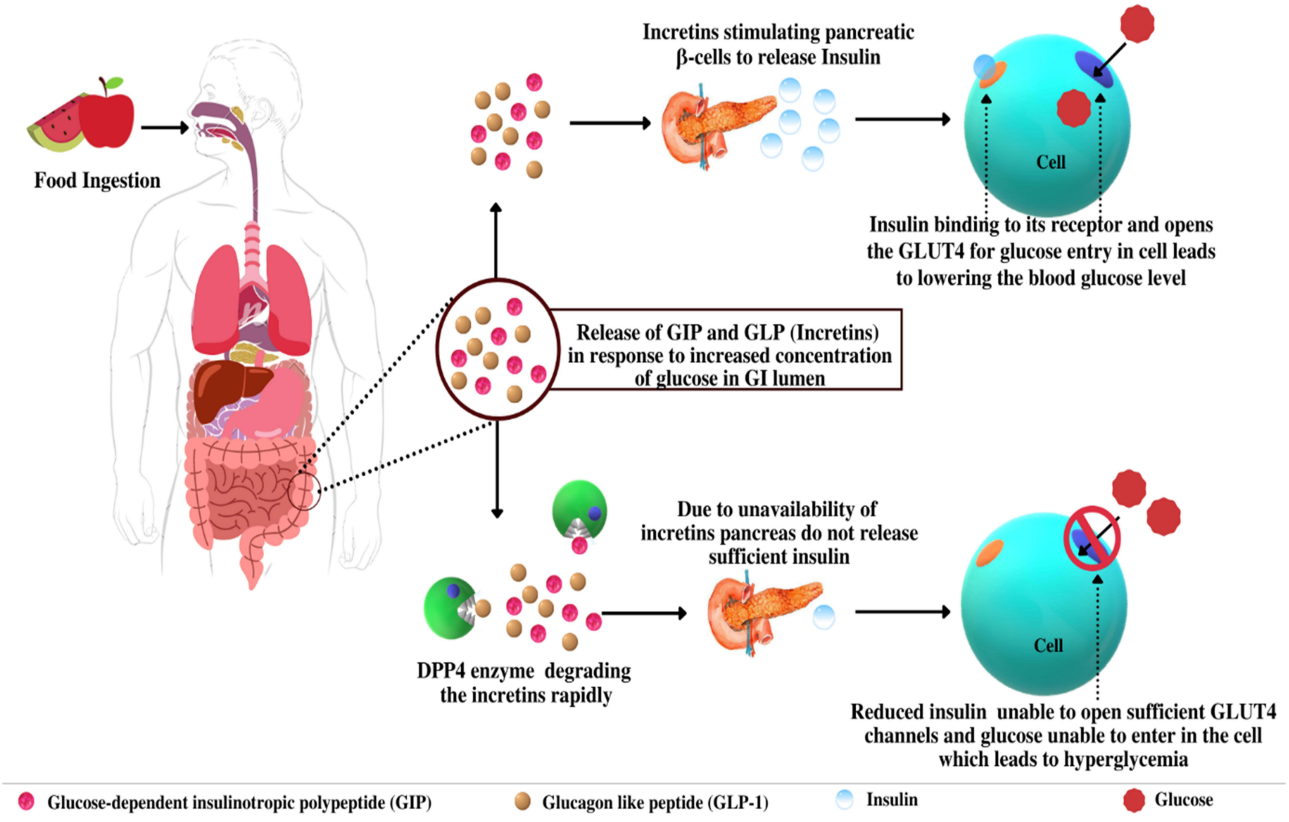

Figure 4 Role of Incretins and DPP-4 in glucose regulation.

DPP-4 Inhibitors in Hepatitis C Virus (HCV)

HCV is a serious public health concern around the world. Consequently, HCV has a high proclivity for causing severe infection, and chronic hepatitis C affects 58 million people worldwide, with about 1.5 million new infections occurring per year as per reports by WHO. This can progress to severe hepatic fibrosis, cirrhosis, and hepatic cancer in the long run. As a result, in developed countries, HCV is a very common reason for liver transplantation.[74] Interferon has always been the cornerstone of HCV treatment for almost two decades. In 1998, ribavirin was added to the medication, and subsequently, in 2001–2002, the interferon (INF) molecule was linked to polyethylene glycol (PEG) to enhance treatment responses.[75,76] IP-10 (interferon-inducible protein of 10 kDa), commonly known as chemokine ligand 10 (CXCL10), is a CXC chemokine that binds to chemokine receptor 3 (CXCR3) and plays a vital role in selecting candidates for T lymphocytes and natural killer cells. IP-10 and other chemokines are secreted by hepatocytes infected with the hepatitis C virus to boost the adaptive and innate immune response.[20] Surprisingly, elevated blood levels of IP-10, a powerful chemoattractant, have been linked to PEG-IFN and ribavirin therapy failure. IP-10 is usually changed by DPP-4, which produces the antagonist version of IP-10 by cleaving two amino acids from the amino terminal portion of IP-10. Antagonist version of IP-10 has the ability to bind to the IP-10 receptor but does not cause signalling. CD8+ T-cells, which express DPP-4, have also been seen in the portal and periportal areas of patients with HCV infection. In hepatocytes, DPP-4 expression is enhanced in patients with HCV infection.[69,77] In patients with HCV infection, a high baseline blood soluble DPP-4 concentration is linked to poor treatment results. The IP-10 and DPP-4 proteins' expression and binding capabilities are affected by genetic differences in the IP-10 and DPP-4 genes.[78,79]

According to lymphocyte subset analysis, HCV attacks CD8+ T-cells; hence, HCV-infected T-cells could be blamed for the elevated blood DPP-4 activation in HCV patients. DPP-4 alters the immune response by cleaving two amino acids from the amino-terminal portion of IP-10 which suppress the immune responses toward the HCV which may lead to more severe hepatic infection.[80,81] Furthermore, Hepatitis-C is related to hyperglycemia and insulin sensitivity, which is linked to the progression of the disease and prognosis because of elevation in DPP-4 level.[82–89] HCV is engaged in the

development of insulin resistance by the disruption of signaling pathway substrate,[90] in addition to hepatic inflammation and steatosis. Furthermore, Hepatitis-C has been linked to higher DPP-4 expression in the intestinal lumen, hepatic portion, and blood.[77,91] Transfection of hepatocyte cell lines with cDNA expressing a portion of the Hepatitis viral non-structural genomic region 4B/5A increases DPP-4 expression.[92] HCV infection may directly upregulate DPP-4 activity, resulting in glucose metabolism impairment.[16,77] Inhibition of DPP-4 is significant in HCV infection as well as in glucose intolerance as successfully shown in Figure 5.

Hence, interferon therapy for HCV eradication lowers serum DPP-4 levels and helps in treating the HCV,[90,93–96] and Sitagliptin treatment dramatically improves HCV-related glucose intolerance.[97,98]

DPP-4 Inhibitors in Non-Alcoholic Fatty Liver Disease (NAFLD)/ Nonalcoholic Steatohepatitis (NASH)

NAFLD is the most prevalent cause of chronic liver disease.[99–102] It is a hepatic expression of metabolic syndrome. Whereas many factors contribute to the formation of NAFLD, elevated blood glucose has been observed, stimulated by DPP-4 expression in hepatoma cells (HepG2), and the amount of liver DPP-4 mRNA activity in the liver is much higher in NAFLD patients than in healthy subjects.[103] Cui et al 2016 conducted a randomized controlled trial for NAFLD by DPP-4 inhibitor (sitagliptin) versus placebo. Researchers randomized, double-blind, placebo-controlled clinical study to compare the effectiveness of sitagliptin (100 mg/day orally) versus an identical placebo for 24 weeks to improve hepatic steatosis as measured by MRI-PDFF (Magnetic Resonance Imaging Proton Density Fat Fraction), which is a proven, precise, and quantifiable biomarker for hepatic steatosis. Fifty patients of NAFLD were randomised to receive sitagliptin and placebo from January 2014 to March 2015. The research included 84 patients in total. The primary outcomes of their study towards the liver fat which is measured by MRI-PDFF, when compared to the placebo group, was not substantially lowered in the sitagliptin group. Sitagliptin was not really substantially superior than placebo for lowering liver fat as evaluated by MRI-PDFF in this randomised, double-blind, placebo-controlled clinical study. Sitagliptin did not outperform placebo in terms of improving supplementary targets such as LDL, AST, ALT, and HOMA IR. Sitagliptin did not markedly reduce fibrosis as determined by MRE, despite the fact that participants in the placebo group had more fibrosis. In the conclusion, it is reported that sitagliptin was shown to be safe but ineffective in lowering liver fat in persons with

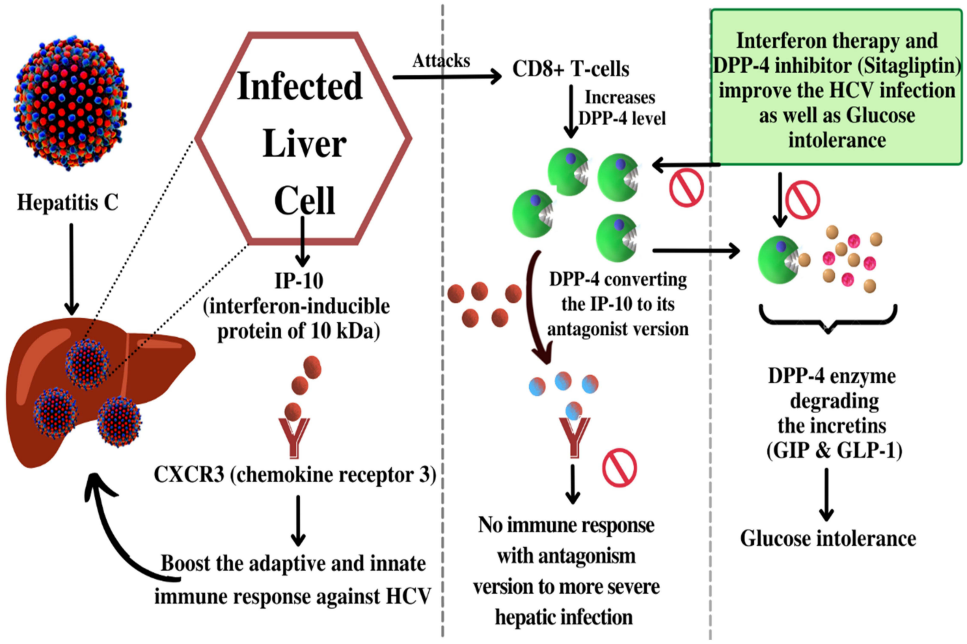

Figure 5 Schematic representation of HCV infected hepatocytes releases IP-10 responsible for an immune response towards HCV infection but DPP-4 level elevated due to CD8+ cells attacked by HCV. Increased DPP-4 converted the IP-10 into an inactive form which suppresses the immune response and on the other hand DPP-4 results in glucose intolerance by degrading incretins. Interferon and DPP-4 inhibitors are found to be significant in both HCV resulting conditions.

NAFLD who were pre-diabetic or diabetic, and this trial was observed for 24 weeks only.[104] On the other hand, Alam et al[105] conducted a randomized controlled trial for the impact of sitagliptin on nonalcoholic steatohepatitis patient's hepatic histological activity and fibrosis which was observed for 12 months in a randomized control study. That randomized controlled research found that using sitagliptin (100 mg daily) for one year, a DPP-4 inhibitor reduces steatosis and swelling in NASH patients. The NAS (score for NASH) in coupled biopsy samples was considerably reduced as a result of these two adjustments. This intervention did not affect fibrosis. The control group's NAS was likewise reduced by steatosis reduction, although hepatocyte ballooning remained the same. The sitagliptin group was shown to have a much larger reduction in steatosis and NAS than the control group. Regardless of diabetes condition, sitagliptin (100 mg once daily) for a year reduces NAS through alleviating steatosis and hepatocyte enlargement. Sitagliptin has a more powerful effect than weight loss. Sitagliptin has identical safety profile to the control. To validate and solidify these findings, future major, double-blind, randomised control clinical studies are recommended. In a study of fructose-fed rats with metabolic syndrome, sitagliptin shown to be reduced liver steatosis, β-cell apoptosis, and insulin sensitivity.[106] Another animal research in Japan found that sitagliptin helps to reduce hepatic steatosis in mice fed a high-fructose diet and prevents the growth of NAFLD by suppressing inflammatory cytokines and the expression levels of genes involved in lipid production in the liver.[107] The study's most important conclusion was that sitagliptin reduced the severity of hepatocyte ballooning hepatic histopathology. Ballooning degradation, which was identified as a characteristic of steatohepatitis, is connected to cytoskeletal damage in NASH and is associated with cell swelling.[108,109] As a result, it is tempting to say that DPP-4 inhibitors may improve histology activity by lowering steatosis and swelling. Another uncontrolled experimental trial from Turkey found a similar histologically verified advantage.[110]

Apart from DPP-4 inhibitors, Glucagon-like peptide-1 receptor agonists (GLP-1 RAs) are a kind of glucose-lowering medication that has been authorized to treat Type 2 diabetes.[111] Large randomized controlled trials on GLP-1 RAs have also consistently shown that these medicines reduce the risk of adverse cardiovascular events, all-cause morbidity, and nephropathy worsening in T2DM patients[112,113] GLP-1 RAs reduce body weight and insulin sensitivity while improving glycemic management.[111] A number of RCTs have recently investigated the putative positive hepatic effects of liraglutide and other long-acting injectable GLP-1 RAs among individuals with NAFLD, regardless of diabetes status. GLP-1 RAs were studied for their effectiveness and safety in treating NAFLD or NASH in people either with or without pre-existing T2DM. Mantovani et al[114] compared and conducted the largest and most up-to-date systematic review and meta-analysis of RCTs that used different GLP-1 RAs (including two new long-acting injectable GLP-1 RAs, such as dulaglutide and semaglutide) for the treatment of NAFLD or NASH, regardless of T2DM status. Treatment given with GLP-1RAs was observed to be related to a substantial improvement in the absolute percentage of liver fat content, as measured by magnetic resonance-based methods, as well as blood liver enzymes (particularly serum ALT and GGT levels), as compared to control or standard therapy. The current meta analysis does not include a detailed examination of the hypothesized molecular pathways via which GLP-1 RAs may help people with NAFLD. However, it is plausible to infer that liraglutide's and other GLP-1 RAs' good effects on individual NASH histologic scores are multidimensional and a result of their combined effects on hyperglycemia or insulin resistance, weight loss, and a direct positive impact on the liver (beyond the reduction in body weight and hyperglycemia). In reality, GLP-1 RAs are effective in the treatment of T2DM and can also help people lose weight (on mean 4–5 kg).[115] GLP-1 RAs are also able to alleviate hepatic steatosis through lowering de novo lipogenesis, boosting fatty acid oxidation, and improving several aspects of the insulin signaling pathways, according to experimental findings based on both human hepatocytes and animal models.[116–120] Furthermore, preclinical NASH investigations have revealed that GLP-1 RAs may lower hepatic inflammation via independent pathways, at least in part, of body weight loss.[121] Obesity could be a reason for NAFLD and for that cause GLP-1 RAs could be a choice, as recent clinical studies have been shown to successfully promote weight loss in diabetic individuals. The existing evidence suggests that weight loss caused by GLP-1R agonism in humans is mostly due to reduced food consumption. GLP-1 (glucagon-like peptide-1) is known as an endogenous peptide produced in the gastrointestinal tract by enteroendocrine specifically by L cells. GLP-1RAs can help with glucoregulation by promoting satiety, delaying stomach emptying, and lowering calorie intake. The only GLP-1RA licensed for the treatment of obesity is liraglutide. Semaglutide's first Phase III clinical trial has finished, and the results indicated a considerable weight loss benefit. GLP-1RAs have been shown in clinical studies to be effective and safe, and they are regarded as potential anti-

obesity medications.[122] On the other side, according to Velija-Asimi et al 2013, it is found that DP-4 inhibitors DPP-4 inhibitors in combination with metformin were related to improved glycaemic control and a decrease in body weight in obese adults with type 2 diabetes.[123]

The increase of intrahepatic triglycerides (TGs) is the major symptom of NAFLD, which affects 75–90% of people with type 2 diabetes.[124,125] NAFLD can proceed to NASH, which is marked by extensive histologic transformation, such as hepatocellular ballooning, lobular inflammation, fibrosis, and an increased risk of hepatocellular carcinoma. Various pharmacotherapies are being explored since insulin resistance, oxidative stress, lipotoxicity, immunology, mitochondrial damage, the cytokine system, and apoptosis are all implicated in the pathophysiology of NASH. Although no medicine is available for the evidence-based therapy of NASH, antidiabetic therapies may be beneficial in individuals who also have diabetes mellitus. Several investigations have found a relationship between DPP-4 and hepatic insulin sensitivity. Upregulation of DPP-4 in hepatocytes is linked to hepatic insulin resistance and liver steatosis as observed in rats,[73] whereas knocking down DPP-4 optimizes insulin sensitivity and lowers lipid buildup in cultured hepatocytes.[126] DPP-4 has also been linked to the occurrence of insulin sensitivity and glucose intolerance in the liver and adipose tissue, according to other research. Obesity and accompanying visceral adipose tissue inflammation cause insulin sensitivity in mice, a process that appears to be driven by increased hepatic DPP-4 production and release, since abolishing hepatocyte DPP-4 expression reduces inflammation and improves insulin sensitivity. DPP-4 is thought to be a new adipokine that affects insulin sensitivity in both autocrine and paracrine ways. DPP-4 release is closely correlated with adipocyte size, suggesting that adipocytes may be a major source of DPP-4.[127] The more fat in the liver, the higher the activation of hepatokine DPP-4, which might lead to NAFLD and subsequently, NASH in a paracrine and autocrine manner. Thus, omarigliptin may inhibit the activity of DPP-4, which is abundantly released from the liver in NAFLD/NASH, preventing the stimulation of adipose inflammation and insulin resistance in the liver.[128] According to Wang et al 2021, study findings show that the major cause of hepatic inflammation like NFκB pathway activation, oxidative stress, and cell apoptosis inhibition reduces hepatic inflammation. In the study, sitagliptin was found to be restricting the DPP-4 activity in hepatocytes reducing NFκB pathway activation and oxidative stress, as well as cell apoptosis, in diabetic conditions, and sitagliptin's ROS cleaning function promotes NFκB pathway deactivation; additionally, sitagliptin can reduce Streptozotocin chronic hepatotoxicity and oxidative stress. Under diabetes circumstances, sitagliptin inhibits DPP4 activity in hepatocytes, resulting in reduced NFκB pathway activation, oxidative stress, and cell death.[122] The inactivation of the NFκB pathway is promoted by sitagliptin's ROS cleansing action and DPP-4 inhibitors are also known for the reduction in body weight in obese adults with type 2 diabetes.[122] But there is vildagliptin, which is also a strong and selective DPP-4 inhibitor that is weight neutral in type 2 diabetic patients in several solotherapy and combined studies. Because of its glucose-dependent mode of action, vildagliptin has a reduced risk of hypoglycemia, which eliminates the "defensive eating" that can emerge with insulin injections or independent glucose-insulin secretagogues. More data show that vildagliptin may affect postprandial lipid and lipoprotein metabolism by decreasing the absorption of triglyceride from the gut and boosting sympathetically triggered lipid mobilization and catabolism in the postabsorptive phase. Additional research into these pathways might offer a molecular foundation for understanding the weight-loss benefits of vildagliptin medication.[129] Vildagliptin is an important DPP-4 inhibitor that may be used for lowering the risk or decreasing hepatic inflammation without body weight reduction.

In reality, hepatic DPP-4 expression and serum DPP-4 activity are linked to hepatic steatosis and fatty liver grading.[130,131] Furthermore, as compared to wild-type rats, DPP-4 deficient animals have lower levels of liver pro-inflammatory and pro-fibrotic cytokines, as well as less hepatic steatosis. These beneficial alterations in lipid metabolism are not caused by changes in glucose metabolism.[132] In individuals with NAFLD, DPP-4 activity in serum and liver specimens correlates with indicators of hepatic injury like blood gamma-glutamyl transferase (GGT) and alanine aminotransferase amounts, but not with fasting blood glucose levels or glycosylated hemoglobin (HbA1c) values, similar to the findings in animal studies. As a result, hepatic DPP-4 expression in NAFLD could be linked to hepatic lipogenesis and liver damage.[133,134] In humans and rodents, a DPP-4 inhibitor has been shown to ameliorate hepatic steatosis.[135] The activity of DPP-4 inhibitors is successfully shown in Figure 6.

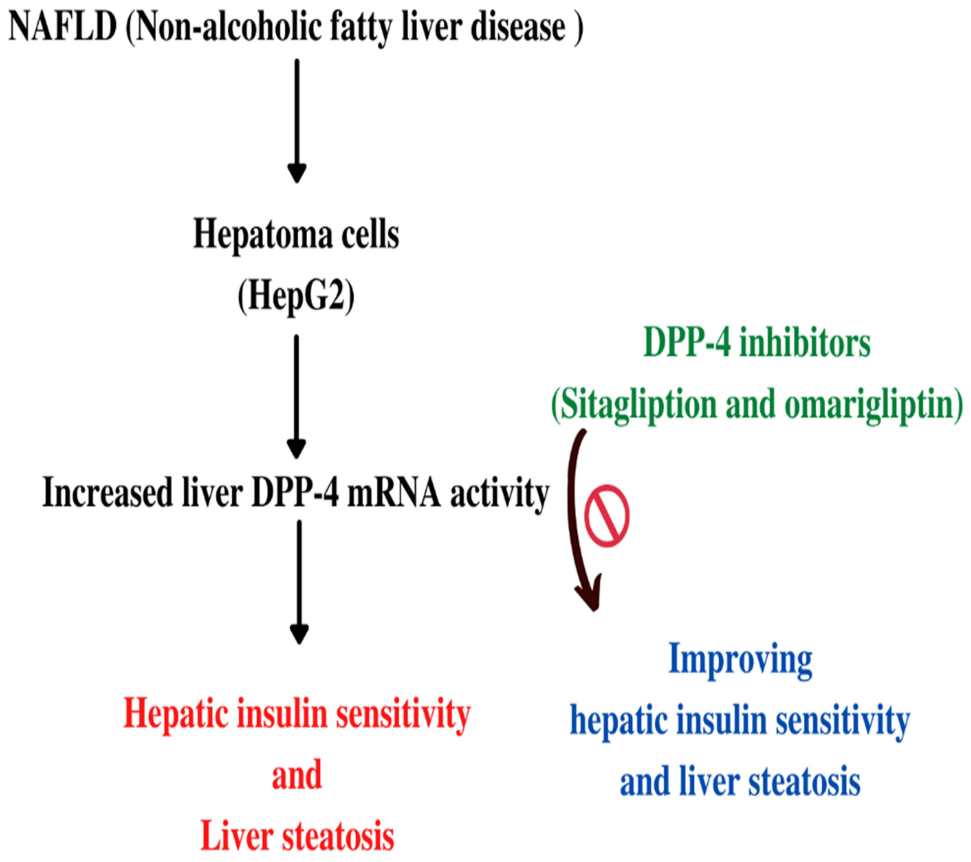

Figure 6 Non-alcoholic fatty liver disease results in an increased level of DPP-4 expression leads to hepatic insulin sensitivity and liver steatosis but sitagliptin and omarigliptin improve the conditions.

A case of refractory fatty liver that was successfully treated with sitagliptin, a DPP-4 inhibitor.[136] In addition, omarigliptin and sitagliptin have been shown to reduce liver enzymes and hepatocyte ballooning in patients with NASH.[110,128] These data suggest that DPP-4 inhibitors may help patients with NAFLD with hepatic damage and glucose intolerance.

DPP-4 Inhibitors in Hepatic Regeneration and Stem Cell

The cirrhotic liver has been shown to have increased hepatic DPP-4 expression.[128,137] Although the consequence of increased DPP-4 expression is unknown, recently showed that human liver stem cells express DPP-4 but not CD34 or CD45, which are markers of hematopoietic stem and endothelial progenitor cells.[138] If we understand the concept of Cell-released chemokines, cytokines, and other growth-modulating substances that elicit their effects through particular receptor-mediated intracellular signaling modulate hematopoietic progenitor cell (HPC) and hematopoietic stem cell (HSC) functions in a paracrine manner.[139] Other progenitor and stem cell types are regulated by these proteins, and also impact the more mature cell's function. On HPCs expressing CD26, inhibiting DPP4 enzymatic activity with short peptides such diprotin A (ILE-PRO-ILE) or VAL-PYR improves chemotaxis to the chemokine stromal cell-derived factor-1 (SDF-1/CXCL12)[140] as well as homing and engraftment of HSCs.[141–143] CXCL12 with a DPP4 truncation lacked chemotactic efficacy but prevented chemotaxis triggered by full-length SDF-1.[140] A pilot clinical trial evaluated the effects of sitagliptin (inhibitor of DPP4 used to treat type 2 diabetes)[144] administration to patients with high-risk hematologic malignancies receiving single-unit cord blood transplants. With the findings that DPP4 has a detrimental effect on CSFs6, which nourish immature cell types in the bone marrow, attempts are being made to change the dosing schedule of sitagliptin to improve the time to engraftment of cord blood.[145] Chemokines are important for degranulation, angiogenesis, and leukocyte trafficking in the immune system,[146] and DPP4 may have

a major impact on the activity of chemokine. DPP4 induces negative feedback by lowering CCL22/MDC activity, similar to its actions on CXCL.[140,147,148] CCL22 purportedly possesses anti–HIV-1 action and attracts activated lymphocytes, dendritic cells, natural killer cells, and monocytes. In CCR4-transfected cells, DPP4-truncated CCL22 fails to desensitize calcium mobilization by full-length CCL22 or thymus and activation-regulated chemokine.[149] HUT-78 T-cell chemotactic activity is reduced by truncated CCL22, which is 100 times less effective than full-length CCL22. As a result, DPP4′s N-terminal truncation of CCL22 has various effects on its multiple immunologic roles. Eosinophils are drawn to allergic inflammation and parasite infections by the CCL11 (eotaxin) and, CC chemokine. When DPP4 truncates it, its chemotactic potency for signaling capability and blood eosinophils through CCR3 are lowered 30-fold.[44] These examples show the importance of DPP4 in infectious processes and inflammatory, as well as in steady-state hematopoiesis. It has been documented that the DPP4-truncated versions of the chemokines studied (CCL2, CCL3, CXCL8/IL-8, and CXCL9) lost their suppressive effect and blocked myelosuppression in vitro and in vivo when compared to their full-length counterparts. The shortened molecule functions as a dominant-negative or competitive inhibitor form of the full-length molecule in both circumstances. This could lead to feedback regulation of their full-length molecules' actions. It's also possible that DPP4 truncation enhances a molecule's stimulatory or inhibitory activity beyond that of the full-length version.[145] It's critical to double-check protein sequences in databases containing potential DPP4 truncation domains on a regular basis to make sure they have not been altered. TGF-, for example, once had a DPP4 truncation site; however, the sequence has since been changed and no longer possesses a DPP4 site. Finally, biochemical and biological (in vitro and in vivo) studies are needed to confirm whether the putative DPP4 truncation sites are true truncation sites for each protein, especially when different alanine, proline, serine, or other potential DPP4 truncation sites are present at the N-terminus of every molecule. If that is the case, it is crucial to figure out whether the abbreviated form's activity differs from that of its full-length counterpart, and if so, how. Overall understanding of the in vitro and in vivo control of various stem, progenitor, and more mature hematopoietic and other kinds of cells might result from such studies. This data might have therapeutic implications.[145]

Through activation of insulin resistance (IR), obesity-related inflammation raises the risk of type 2 diabetes mellitus (T2DM), obstructive sleep apnea syndrome (OSAS), and polycystic ovary syndrome (PCOS).[150] In obesity-related NAFLD, IR is nearly universally found, leading to the development of the metabolic syndrome and hepatocarcinoma.[151] Stem cell growth factor-beta (SCGF-β) has been shown to have activity on macrophage/granulocyte progenitor cells.[152,153] C-reactive protein (CRP) levels were found to be elevated only in one-third of obese patients in the investigation, indicating a link with SCGF. The study characterizes itself by the prediction of homeostatic metabolic assessment (HOMA) values by SCGF levels, possibly mediated by indicators of inflammation, offering some insight on processes inducing/worsening IR in male patients with obesity-related NAFLD. M-CSF, TNF-, IL-12p40, and IL-6, among other pro-inflammatory cytokines, were not linked with HOMA values, with the exception of IL-6, which predicted a reduced chronic inflammation state. The small rise in CRP levels supports this notion. According to the study of Tarantino et al 2020, suggest that barely raised CRP levels might make IL-10 more accessible in an attempt to partially decrease inflammation, the major cause of IR, in line with data that CRP affects the anti-inflammatory or pro-inflammatory balance, exacerbating inflammation. In this regard, we would like to call attention to our results, which include the presence of IR in almost half of the obese individuals, increased levels of IL-10, and IL-12p40′s defensive response. SCGF- serum concentrations might also be due to hematopoietic stem or progenitor cells' limited autocrine/paracrine activity. It is thought that by switching M1 to M2, inflammation could be reversed and IR reduced. Even though our median HOMA values overlapped according to gender, individuals with a more prominent HOMA had a greater frequency of moderate-to-severe steatosis than those with a HOMA below the median. The finding that SCGF levels solely predicted the severity of hepatic steatosis in men might indicate that these patients' obesity influences their inflammatory state and/or immune system. As a result, only males' CRP and IL-6 levels predicted SCGF-concentrations. These findings support the observation that SCGF levels solely predict IR, as measured by HOMA, in males. CRP's mediating involvement is conceivable when we consider its functional role in inflammation. In summary, this study is characterized by the estimation of HOMA values by SCGF levels, which is likely mediated by inflammation, providing insights on processes worsening IR in male patients having

obesity-related NAFLD.[154] As a result, DPP-4 is a particular marker of adult hepatic stem and progenitor cells, suggesting that it may play a role in liver regeneration in chronically inflamed patients. CXCL12/SDF-1 is a chemokine that promotes the homing of hematopoietic stem cells (HSCs) and is critical for hepatic regeneration.[155,156] CXCL12/SDF-1 is a DPP-4 target peptide, and inhibiting cell-surface DPP-4 activity promotes CXCL12/SDF-1 directed chemotaxis, homing, and engraftment in HSC/hematopoietic progenitor cell populations. As a result, inhibiting DPP-4 might be a good way to improve the efficacy and success of HSC/hematopoietic progenitor cell transplantation.[157] DPP-4 suppression also increases the number of progenitor cells, and DPP-4 inhibition can stabilize endogenous CXCL12/SDF-1, which could be a promising technique for increasing the sequestration of regenerative stem cells.[158]

DPP-4 Inhibitors in Hepatocellular Carcinoma

Breast cancer,[159,160] malignant mesothelioma,[161] lung cancer,[162] and squamous cell laryngeal carcinoma[163] are all known to have increased DPP-4 expression. Increased DPP-4 expression is also found in liver tissues and serum from rats[164] and humans with hepatocellular carcinoma (HCC).[165]

Higurashi et al (2016) conducted a multicentre double-blind, placebo-controlled, randomized Phase 3 trial for the chemoprevention of metachronous colorectal adenoma or polyps in post-polypectomy patients without diabetes and it is observed that non-diabetic patients were given a small dose of metformin for a year with no side effects. After polypectomy, a small dose of metformin decreased the prevalence and quantity of metachronous adenomas or polyps. Metformin shows the potential to prevent colorectal cancer through chemoprevention. However, further large-scale, long-term studies are required to draw definitive results.[166]

Kawakita et al (2021) observed the potential influence of DPP-4 inhibitors and DPP-4 on cancer with diabetes and states that there is currently no obvious link between DPP-4 inhibitors and cancer incidence or prognosis in diabetic individuals, according to available clinical evidence. However, the safety profile of a DPP-4 inhibitor (which is the same as different anti-diabetic medications) on cancer development or recurrence has yet to be shown. The results suggested for further mechanistic studies into the relationship between DPP-4 inhibitors and cancer biology, particularly in diabetic situations, are an important study subject in both diabetes and oncology.[167] Zhao et al 2017 worked on a meta-analysis of randomized clinical trials on DPP-4 inhibitors and cancer risk in patients with type 2 diabetes and there were 72 studies in all, with 35,768 and 33,319 patients recruited in the DPP-4 inhibitors and comparator medicine trials, respectively. In comparison to the usage of other active medicines or placebo, no significant connections between DPP-4 inhibitor use and cancer development were found. The findings were similar in pre-defined subgroups stratified by DPP-4 inhibitor type, cancer kind, comparative medication, trial duration, or baseline characteristics. The findings of this meta-analysis reveal that people with type 2 diabetes who take DPP-4 inhibitors have no increased risk of cancer than people who take a placebo or other medicines. Wilson et al 2021 provide clear evidence data that the currently authorized medication sitagliptin (DPP-4 inhibitors) can boost antitumor immunity in a syngeneic ovarian cancer mouse model, lowering metastatic burden and lengthening longevity. Our findings suggest a method for improving immune responses in ovarian cancer patients, as well as a justification for using DPP4 inhibitors as a fast translatable 2nd line therapy for this illness.[168]

According to Hsu et al 2021, DPP-4 inhibitors can lower the incidence of hepatocellular carcinoma in individuals with chronic hepatitis C infection with type 2 diabetes. In this study, individuals with type 2 diabetes and persistent HCV infection who used DPP-4 inhibitors had a decreased risk of HCC. DPP-4 inhibitors were associated with a greater incidence of HCC-free patients. This suggests that DPP-4 inhibitors may help people with type 2 diabetes and persistent HCV infection avoid developing HCC. DPP-4 inhibitors may be used as a second-line treatment after metformin for individuals with type 2 diabetes with persistent HCV infection.[69]

DPP-4 inhibition suppresses tyrosine kinase in human hepatoma cells, resulting in anti-apoptotic effects.[165] Recently, a case has been discussed in which a patient with HCV-related chronic hepatitis experienced remarkable HCC reduction following four weeks of treatment with a DPP-4 inhibitor (Figure 7). Although it is unclear whether the DPP-4 inhibitor is directly involved in the regression of HCC, a significant invasion of CD8+ T-cells around the HCC tissue was observed, suggesting that the DPP-4 inhibitor may have improved the immune response, which has

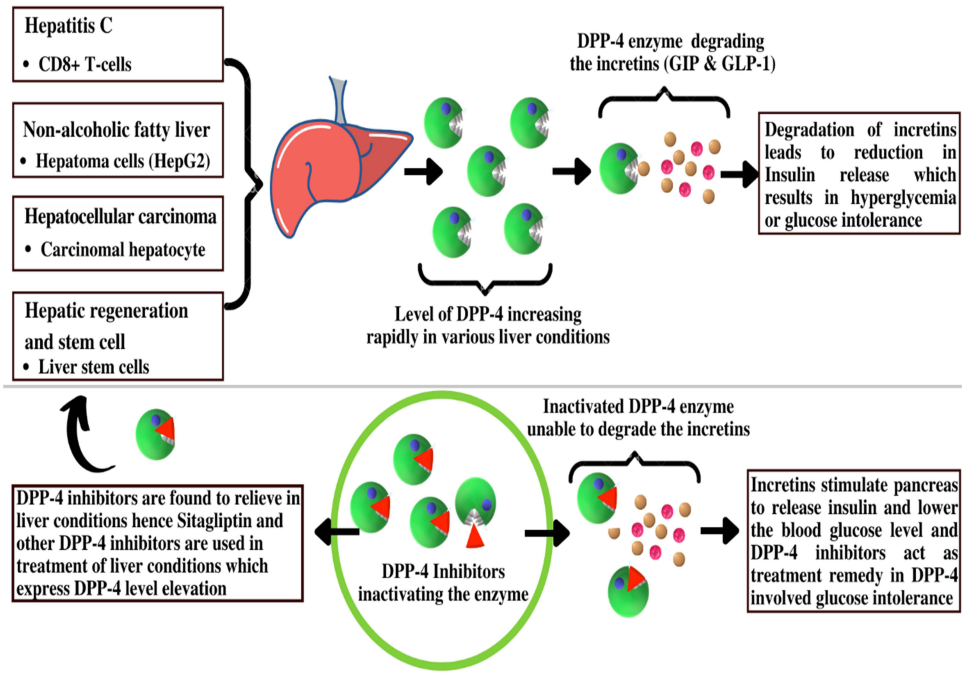

Figure 7 Liver diseases cause an increase in DPP-4, which causes glucose intolerance and DPP-4 inhibitors lead to relief in glucose intolerance as well as in liver conditions.

been compromised by chronic HCV infection.[169] Whereas treatment with exogenous insulin or sulfonylureas raises the risk of HCC,[85] treatment with a DPP-4 inhibitor had no tumor-promoting effects in mice.[170] As a result, a DPP-4 inhibitor may have a safe effect on HCV-related HCC through modulating immunity.

This review discussed the various liver conditions and glucose intolerance management with DPP-4 inhibitors. The summarizing table with the mechanism of action and treatment of liver conditions associated with DPP-4 is given in Table 2.

DPP-4 elevation could be considered a biomarker for diabetes and is a very interesting molecule in understanding the relationship between diabetes and liver or other organs, and inhibition of DPP-4 could help to reduce the risk of its associated diseases but, on the other hand, DPP-4 inhibitors have some negative aspects. DPP-4 inhibitors have been linked to an increase in gastrointestinal side effects in 24-week research, 1091 T2DM patients were randomly assigned to different combinations of sitagliptin and metformin.[173] There have been a number of instances of allergic responses occurring spontaneously in people using sitagliptin and angioedema has also been documented with DPP-4 inhibitors, usually commonly within the first three months of therapy, with some responses occurring even before the first dosage.[174–176] As per the study design of saxagliptin (2.5mg/day v/s 5mg/day v/s 10mg/day) with placebo on metformin for 24 weeks revealed that skin disorders, nasopharyngitis, headache, sinusitis, urinary tract infection, and arthralgia are the adverse effects produced by saxagliptin which are in high proportion than the placebo.[176] Alogliptin versus placebo (Population 5380 and duration is 18 months) study showed the adverse effects of alogliptin at more proportion than placebo such as acute and chronic pancreatitis, angioedema, malignancy, renal dialysis, and hypoglycemia but without a comparison of proportions of alogliptin and placebo showed non-fatal myocardial infarction or non-fatal stroke.[177] Similarly, other DPP-4 inhibitors also showed some side effects such as musculoskeletal disorders, infections (immune-related disorders such as irritable bowel syndrome, arthritis, and multiple sclerosis because of their potential influence on immunological function), nervous system (Headache and dizziness), Fertility (A 39-year-old physician started on sitagliptin, he had issues with spermatogenesis, according to a case study), and Blood effects (increase in white blood cell count).[178]

Table 2 Various Mechanisms of Action and Management of Some DPP-4-Associated Liver Diseases

Disease	Area of Concern	Mechanism of Action	Management/Reduce the Risk of Concern Disease	Reference
Hepatitis C	CD8+ T-cells	HCV attacks CD8+ T-cells, hence HCV-infected T-cells could be blamed for the elevated blood DPP-4 activation and DPP-4 inactivate of the incretins which lead to hyperglycemia.	Interferon therapy for HCV and Sitagliptin	[80,81]
Non-alcoholic fatty liver	Hepatoma cells (HepG2)	Elevated blood glucose is stimulated by DPP-4 expression in hepatoma cells (HepG2), and the amount of liver DPP-4 mRNA activity in the livers. Hepatic DPP-4 expression and serum DPP-4 activity are linked to hepatic steatosis and fatty liver grading. DPP-4 amount elevation causes glucose intolerance	Sitagliptin	[103,130]
Hepatocellular carcinoma	Carcinomal hepatocyte	Increased DPP-4 expression is also found in liver tissues and serum from rats and humans with hepatocellular carcinoma (HCC). DPP-4 inhibition suppresses tyrosine kinase in human hepatoma cells, resulting in anti-apoptotic effects. Recently, a patient with HCV-related chronic hepatitis experienced remarkable HCC reduction following four weeks of treatment with a DPP-4 inhibitor.	DPP-4 inhibitors like Sitagliptin, saxagliptin, linagliptin, and alogliptin.	[165,171,172]
Hepatic regeneration and stem cell	Liver stem cells	CXCL12/SDF-1 is a chemokine that promotes the homing of hematopoietic stem cells (HSCs) and is critical for hepatic regeneration. CXCL12/SDF-1 is a DPP-4 target peptide, and inhibiting cell-surface DPP-4 activity promotes CXCL12/SDF-1. As a result, inhibiting DPP-4 might be a good way to improve the efficacy and success of HSC/hematopoietic progenitor cell transplantation. DPP-4 suppression also increases the number of progenitor cells, and DPP-4 inhibition can stabilize endogenous CXCL12/SDF-1 which also helps in the reduction of hyperglycemia.	DPP-4 inhibitors	[155,157]

Conclusion

In glucose regulation, the role of incretins (GIP & GLP-1) is very important. They are released from the GIT lumen in response to the increased level of glucose during absorption and then stimulate pancreatic beta-cells to release insulin which lowers the blood glucose level by enhancing the entry of glucose in the cell through the GLUT4 channel and the cell utilizes the glucose to form energy. But there is an enzyme that inhibits this process by degrading the incretins and creating low availability of incretins which leads to reduced signaling towards pancreatic β-cells to release insulin resulting in an increased level of blood glucose as glucose remains in the blood, unable to enter in the cell through GLUT4. Apart from that, it is commonly observed that in various liver disorders such as hepatitis C, Non-alcoholic fatty liver, hepatocellular carcinoma, hepatic regeneration, and stem cell the serum level of DPP-4 is increased and leads to glucose intolerance. It is observed and reported that DPP-4 inhibitors are commonly used as a reliever in glucose intolerance and diabetes and have potential activities to improve liver conditions also. Hence, DPP-4 inhibitors like Sitagliptin could be a choice of drug in DPP-4-associated glucose intolerance because of various liver conditions and also in the therapy of liver conditions.

Abbreviations

GIP, Glucose-dependent insulinotropic peptide; GLP, Glucagon-like peptide; VIP, Vasoactive intestinal peptide; PACAP-38, Pituitary adenylate cyclase-activating polypeptide-38; GRP, Gastrin-releasing peptide; NPY, Neuropeptide Y; RANTES, Regulated upon activation; CCL, Chemokine (C-C motif) ligand; CXCL, Chemokine (C-X-C motif) ligand;

SDF-1, Stromal-derived factor-1; MDC, Macrophage-derived chemokine; MIg, Monokine induced by gamma interferon; IP-10, Protein 10 from interferon (γ)-induced cell line; GHRH, Growth hormone-releasing hormone; I-TAC, Interferon-inducible T-cell α chemoattractant; LHα, Leutinizing hormone α chain; IGF-1, Insulin-like growth factor-1; CGRP, Calcitonin-related peptide; hCGα, Human chorionic gonadotropin α subunit.

Disclosure

The authors declare no conflicts of interest in relation to this work.

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Review Article | Open Access

A Revolutionary Blueprint for Mitigation of Hypertension via Nanoemulsion

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Abstract

Hypertension is one of the most important causes of mortality, affecting the health status of the patient. At the same time, hypertension causes a huge health and economic burden on the whole world. The incidence and prevalence of hypertension are rising even among young people in both urban as well as rural communities. Although various conventional therapeutic moieties are available for the management of hypertension, they have serious flaws such as hepatic metabolism, reduced dose frequency, poor aqueous solubility, reduced bioavailability, and increased adverse effects, making the drug therapy ineffective. Therefore, it is required to design a novel drug delivery system having the capability to solve the constraints associated with conventional treatment of hypertension. Nanotechnology is a new way of using and manipulating the matter at the molecular level, whose functional organization is measured in nanometers. The applications of nanotechnology in the field of medicine provide an alternative and novel direction for the treatment of cardiovascular diseases and show excellent performance in the field of targeted drug therapy. Various nanotechnologies based drug delivery systems, such as solid lipid nanoparticles, nanosuspension, nanoemulsion, liposome, self-emulsifying systems, and polymeric nanoparticles, are available. Among them, nanoemulsion has provided a niche to supplement currently available therapeutic choices due to numerous benefits like stability, ease of preparation, enhanced drug absorption, reduced hepatic metabolism, increased dose frequency, enhanced bioavailability, and encapsulation of hydrophilic as well as hydrophobic drugs. This present review provides an in-depth idea about progression in treatment of

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nanoemulsions for treatment of hypertension, recent patents for drug-loaded nanoemulsions meant for hypertension, and marketed formulations of nanoemulsions for hypertension.

1. Introduction

Hypertension is a severe medical condition that raises the threat of brain, heart, and kidney disorders dramatically specified by the perpetual high pressure in the blood vessels [1]. The systolic BP (the pressure exerted by the arterial walls upon the contraction of the heart) to the diastolic BP (the pressure exerted on the arterial walls upon the relaxation of the heart) ratio is a typical way to express blood pressure [2]. It is a serious public health concern and one of the leading causes of death globally. According to World Health Organization, approximately 1.3 billion people globally are affected by hypertension, with the majority (two-thirds) residing in low- and middle-income nations. In a survey conducted in the year 2015, it was shown that one in four women and one in five men are suffering from the problem of hypertension. It was estimated that only about one in every five patients with hypertension has their condition under control, and about 9 million deaths globally are attributed to hypertension globally. The worldwide noncommunicable disease target is to reduce the pervasiveness of hypertension by 25% between 2010 and 2025 [3]. A variety of conventional therapeutic agents acting through various mechanisms are available for the treatment of hypertension but suffers from various constraints like poor aqueous solubility, reduced bioavailability, hepatic metabolism, dose frequency, lack of organ targeting, and higher adverse effects, which can be defeated by the development of advanced systems for drug delivery [4, 5]. Advanced drug delivery systems involve the development of a nanotechnological technique, which is a rapidly developing sophisticated scientific field that encompasses a wide range of disciplines such as chemistry, physics, and biology, as well as unique nanodimension structures with therapeutic applications in pharmacology and the biomedical field [6–8]. Many researchers and scientists are interested in the development and standardization of nanoscale drug delivery systems for a variety of reasons. The nanodimension has a variety of features, including optical, magnetic, and structural surface area ratios, making it a fascinating topic of research in every aspect. It is used as a nanocarrier and a nanoadsorbent and functions as a nanocarrier of therapeutic agents, proteins, or probes, especially because the surface area of nanoscale therapeutics or devices is high [9–11]. These nanoscale approaches include solid lipid nanoparticles, nanoemulsion, nanosuspension, nanoparticles, liposomes, and self-emulsifying systems, among nanoemulsion seems to be a promising and exciting approach to address the constraints of conventional treatment employed for hypertension.

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system because of their extreme micro droplet size with large surface area. They are superior to microemulsions as they avoid the problems associated with microemulsions like coalescence, flocculation, or inherent creaming. Due to various advantages, nanoemulsion drug delivery systems are used in a variety of dosage forms (creams, sprays, foams, solutions, etc.) and result in widespread adoption of them in the pharmaceutical business [12–17]. The current article considers the constraints associated with conventional antihypertensive therapeutics, as well as the significance of the oral nanoemulsion drug delivery method in overcoming the constraints and improving hypertension treatment.

2. Hypertension

Hypertension is one of the most important public health issues, widely acknowledged as the leading cause of global illness burden. It is a lifestyle illness that can be effectively treated by combining a healthy diet with frequent physical activity and maintaining a healthy weight [18]. It is a silent killer because no symptoms are visible in the early stages until a serious medical crisis such as a heart attack, stroke, or chronic renal failure occurs [19]. Because the majority of people are unaware of high blood pressure, the only way to detect it is through measurement. Although the majority of people suffering from hypertension are asymptomatic, some suffer from vertigo, headache, vision alteration, or fainting episodes [20, 21]. A combination of various factors persuading to hypertension and these factors fluctuate from country to country, and even within a country, there are differences between urban and rural communities [22]. When compared to their rural counterparts, city dwellers are more susceptible to certain ailments. According to the National Family Health Survey, India, the prevalence of hypertension was 10.5% in Uttar Pradesh's metropolitan areas and the prevalence of the same issue was 8.3% in rural areas, indicating the higher prevalence of hypertension in urban areas than in rural areas. Quick urbanization, an ageing population, mechanization, changing lifestyles, and dietary changes all contribute to a web of risk factors that entangles people leading to hypertension [20]. The vessels transport blood from the heart to all regions of the body. The heart pumps blood into the veins every time it beats. The force of blood pushing against the walls of blood vessels (arteries) as it is pumped by the heart causes blood pressure [23, 24]. The higher the pressure, the more difficult it is for the heart to pump causing hypertension as depicted using Figures 1(a) and 1(b), and the mechanism responsible for an increase in blood pressure causing hypertension is represented by Figure 2. The normal blood pressure is 120 mmHg (systolic pressure) and 80 mmHg (diastolic pressure), and beyond it, the

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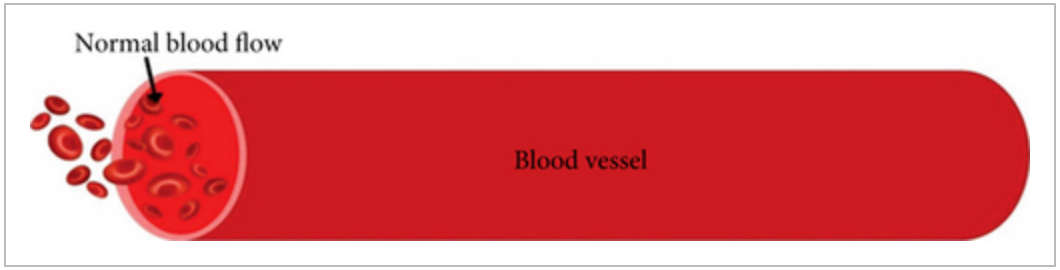

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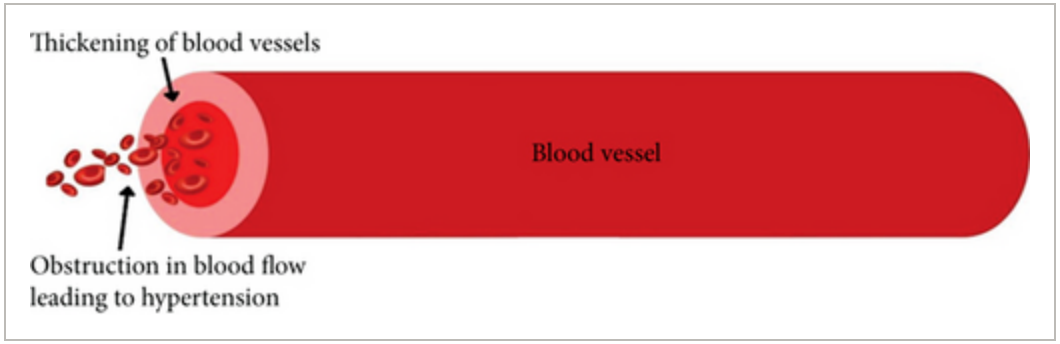

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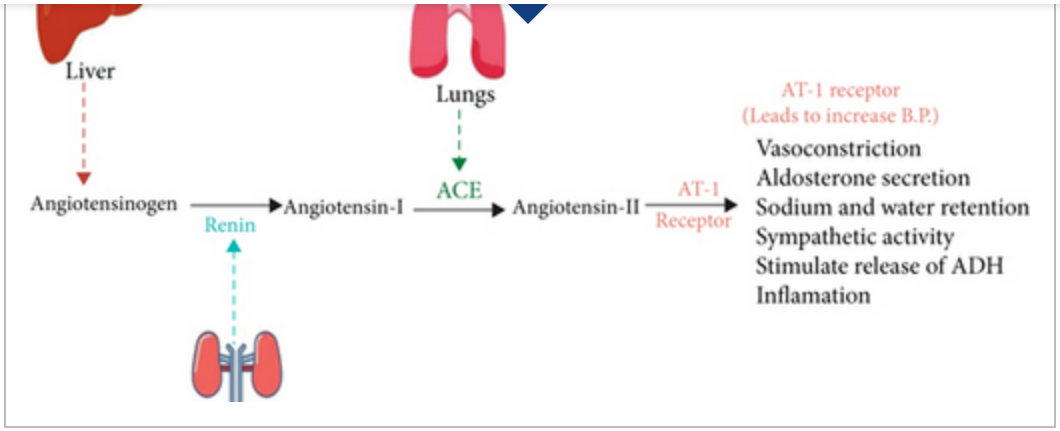

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2.1. Progression in Drug Treatment for Hypertension

Various drug molecules having different mechanisms are available for the treatment of hypertension. Pentaquine was the first drug molecule produced to treat hypertension in 1946; however, it had several side effects and had limited therapeutic efficacy. After this, hexamethonium was introduced in the early 1950s, and while it was effective, it was inconvenient to use [4]. When Veratrum was introduced, it was highly toxic, along with the quick onset of action [26]. Hydralazine was developed immediately after the negative effects of ganglionic blockers, and it is now rarely recommended. Because of its negative effects, such as depression and impotency, reserpine, the most successful medicine developed at the time, was also abandoned [27]. The contemporary age of hypertension therapy began in 1960 with breakthrough medications such as diuretics and β-blockers, which are now frequently prescribed. Drugs blocking calcium channels, inhibiting angiotensin-converting enzymes, and blocking angiotensin were originally developed in the 1990s and are now utilized as first-line therapy, either alone or in combination. The development of various newer drugs for hypertension has been aided by a rigorous consideration of the renin angiotensinogen aldosterone system. There has been significant progress in the development of innovative treatments, one of which has a target that is also related to the renin angiotensinogen aldosterone system [28, 29]. The unique targets that have

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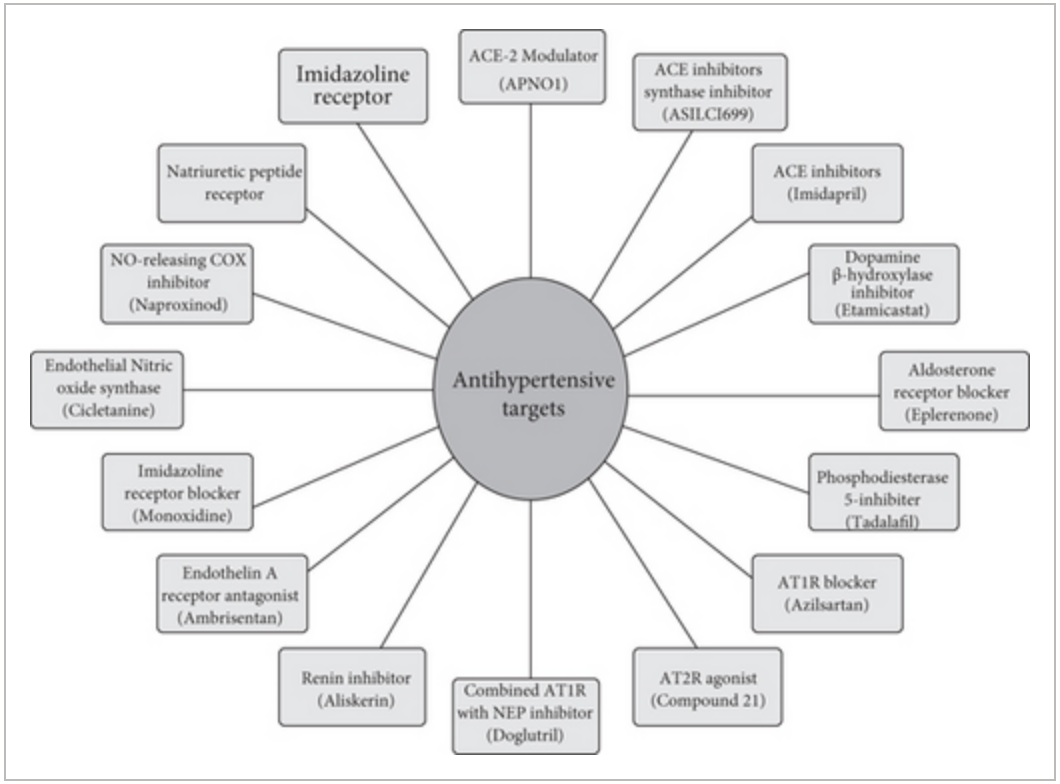

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2.2. Constraints Associated with Antihypertensive Drugs

In general, a drug's solubility and its permeability are critical parameters contributing to the oral absorption of the drugs. For a drug to exhibit higher oral absorption, both its solubility and its permeability must be higher. The drugs belonging to BCS class II exhibit poor aqueous solubility, contributing to the low bioavailability of the drugs [30, 31]. The majority of the antihypertensive drug molecules have poor aqueous solubility, leading to low bioavailability [13, 32]. Various antihypertensive drugs having poor bioavailability are summarized in Table 1, showing the class of drug, its aqueous solubility, permeability, and

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1. Drugs used in the treatment of hypertension along with their characteristics.

Class	Drug	Aqueous solubility	Permeability (log *P*)	Bioavailability	Ref.
Calcium channel blockers	Verapamil	7 mg/ml	3.8	10-20%	[33]
	Felodipine	7.15 *μ*g/ml	4.36	15%	[34]
	Nisoldipine	5.7 *μ*g/ml	3.1	Less than 5%	[35]
	Nitrendipine	2 *μ*g/ml	3.59	10-20%	[36]
	Amlodipine	75.3 *μ*g/ml	2.22	64%	[37]
	Nifedipine	20 *μ*g/ml	2.20	45-56%	[38]
AT1 receptor antagonist	Valsartan	0.1 mg/ml	5.8	Less than 25%	[39]
	Candesartan	$5*10^{-5}$ mg/ml	6.1	40%	[40]
	Irbesartan	Less than 1 mg/ml	4.5	60-80%	[41]
	Telmisartan	0.09 mg/ml	7.7	42%	[42]
	Olmesartan	7.42 *μ*g/ml	3.97	26%	[43]
β-Blockers	Atenolol	1.33 mg/ml	0.16	50-60%	[44]
	Metoprolol	50 mg/ml	2.15	50%	[45]
	Acebutolol	200 mg/ml	1.53	40%	[46]
	Carvedilol	0.583 *μ*g/ml	4.1	23%	[47]

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Drug delivery systems can be characterized as techniques for delivering therapeutic substances into the body [50]. In ancient times, drug delivery to treat hypertension was accomplished by grinding medicinal plants, leaves, or roots and inhaling the smoke of burning medicinal herbs. But these rudimentary techniques of drug delivery lacked a fundamental requirement in drug delivery: consistency and uniformity [51]. Hence, in the last eighteen and early nineteenth centuries, this resulted in the invention of various drug delivery systems for hypertension, which include pills, tablets, capsules, emulsions, suspensions, troches, lozenges, syrups, and various other systems, collectively known as conventional drug delivery systems [52]. These conventional drug delivery systems imposed various drawbacks: reduced bioavailability, lack of site-specificity, higher adverse effects, hepatic metabolism, a requirement for a large dose, fluctuation in steady-state concentration, and additionally the poor aqueous solubility of 90% of newly developed therapeutic moieties, prompting modification in conventional drug delivery systems and the invention of nanotechnology-based techniques [53, 54]. Nanotechnology-based techniques include liposomes, lipid-based nanoparticles, nanoemulsion, polymeric nanoparticles, dendrimers, and polymeric micelles, but nanoemulsion appears to be an exciting and promising drug delivery system to target the drugs in treatment of hypertension [55–57].

3. Nanoemulsion as Drug Delivery System

Nanoemulsion may be defined as an isotropically clear, thermodynamically unstable colloidal dispersion made up of two immiscible phases along with surfactants and cosurfactants to produce a single phase [58]. Every droplet of nanoemulsion exhibits a diameter of 10 to 200 nm which provides various benefits over conventional as well as other modern approaches like an encapsulation of large quantity of the drug, encapsulation of hydrophilic as well as lipophilic drugs, enhanced drug absorption owing to reduced particle size, improved stability, ease of manufacturing, targeting to particular organ or tissue, improved bioavailability, improved aqueous solubility, delivery into several dosage forms, delivery into the body through various routes, substitute for liposomes or another vesicle, improved drug efficacy leading to reduced adverse effects, delivery of peptides prone to enzymatic degradation in GIT, improved drug permeation through the skin, and lack of limitations of macro emulsions like flocculation, creaming, and sedimentation, as represented by Figure 4 [59–64]. Depending on the presence of a disperse phase, the nanoemulsion can be classified into three categories: oil in water (O/W), water in oil (W/O), and multiple emulsion (W/O/W and O/W/O) [65, 66]. Figure 5 depicts the structure and composition of water in oil (W/O) and oil

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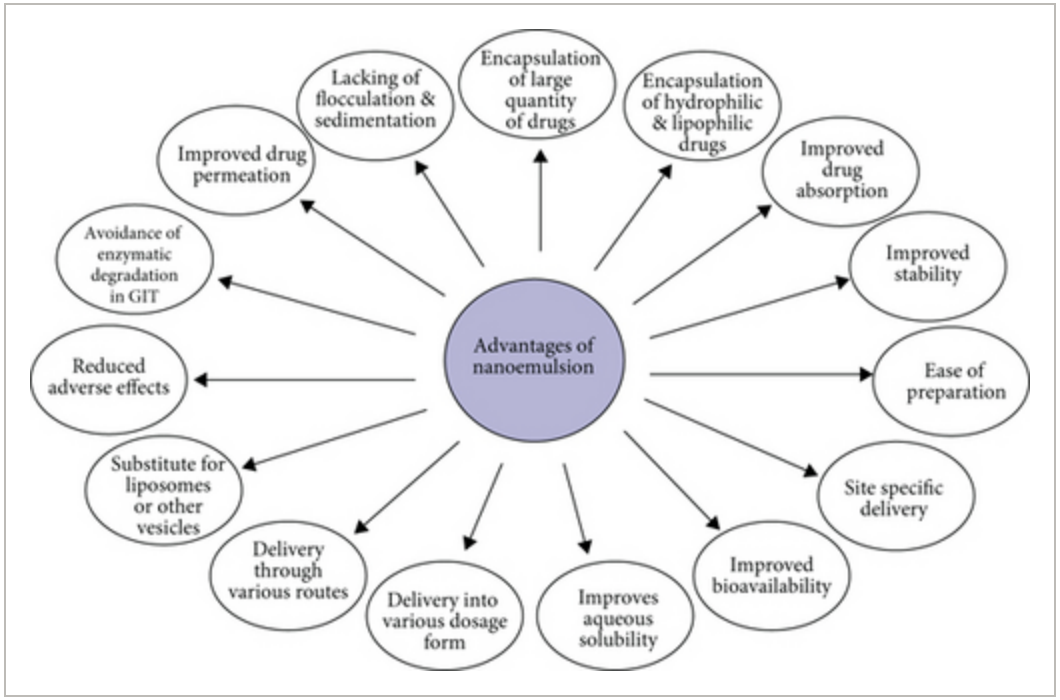
lipid/oil phase, surfactant, and cosurfactant summarized in Table 2 [68, 69].



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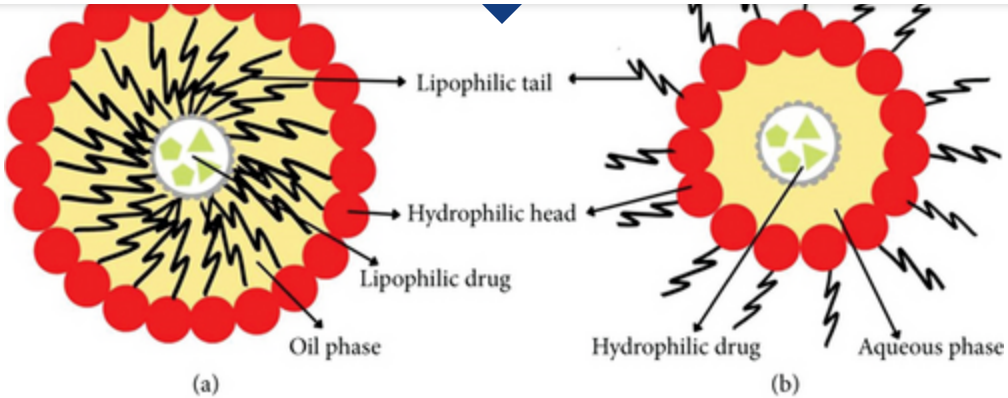

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2. Summarized lipid/oil phase, surfactant, and cosurfactant used for nanoemulsion having antihypertensive drugs.

Type of excipients	Examples
Lipid/oil phase	Soybean oil, coconut oil, seasame oil, cottonseed oil, rice bran oil, Captax 355, safflower oil, Captex 8000, Myritol 318, Witepsol, isopropyl myristate, triacetin, Capryol 90, Castor oil, Sefsol-218, rapeseed oil, olive oil, peanut oil, whale oil, shark liver oil, linseed oil, palm kernel oil, corn oil, jojoba oil, citrus seed oil, almond oil, theobroma oil, ethyl palmitate, octamethyltrisiloxane, hexamethyl disiloxane, fatty ester
Surfactants	Labrafil, Cremophor EL, Lauroglycol 90, Tween 80, Tween 60, Tween 20, Span 80, Span 60, Span 40, Span 20, sodium dodecyl sulfate, lecithin, poloxamers, Labrasol
Cosurfactant	ethanol, propylene glycol, n-butanol, isopropyl alcohol, propanolol, Carbitol, polyethylene glycol 400, Transcutol

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It is reported that the majority of the antihypertensive drugs have low aqueous solubility, which is attributed to the low bioavailability of the drugs [70]. The advancement in drug delivery systems for antihypertensive drugs is nanotechnology, which will be either nanoparticles or nanoemulsion. The mainstay of the nanoemulsion approach in the management of hypertension is mainly achieved using the oral administration of antihypertensive drugs. Scientists and researchers will be more interested in formulations based on the nanoscale level in the management of hypertension [71, 72]. Since hypertension is associated with various consequences, researchers and formulation scientists are interested in using nanotechnology-based drug delivery systems for consequences associated with hypertension [73]. The administration of nanoemulsion through the oral route is most convenient to deliver the drug than other routes due to various benefits like self-administration, ease to administration, dose accuracy, patient compliance, and cost-effectiveness; primarily, oral administration is preferred [74–76]. In earlier times, various scientists have reported considerable improvement in the oral bioavailability of antihypertensive drugs having poor aqueous solubility and high hydrophobicity using the nanoemulsion technology summarized in Table 3. On administration of poorly aqueous soluble drugs using nanoemulsion, significant improvements in pharmacokinetic parameters (Cmax and AUC) were reported, demonstrating the pharmacokinetic benefit of the nanoemulsion over conventional technology [77–79]. Upon oral ingestion, the nanoemulsions enter the gastrointestinal system (GI tract) and are exposed to a variety of environmental variables [80]. In response, gastric lipase is secreted in the gastrointestinal system due to stimulation of the lipid sensing mechanism, enabling the fractional digestion of the lipid layer of nanoemulsion followed by the yielding of simpler diglycerides, monoglycerides, and free fatty acids [81, 82]. The lipase activity is accelerated by the nanoemulsion droplet's small size. The digestion of the lipid component of the nanoemulsion liberates the drugs, followed by nanoprecipitation [83]. In some cases, the drug will simply partition out of the lipid component into the surrounding aquatic environment. Lipids and lipid digested products in the gastrointestinal tract promote bile production and slow down gastrointestinal motility [84]. Bile components act as endogenous surfactants and may create colloidal structures termed "mixed micelles," which promote nanoemulsion solubilization. Thus, bile and preexistent mixed micelles enable the solubilization of freed drug even more and transport it through the unstirred aqueous diffusion layer for absorption [82]. Paracellular or transcellular channels along with M-cells in Peyer's patches also provide intact absorption of the drugs through the oral route due to endocytosis followed by drug transport into intraepithelial spaces as depicted in Figure 6. Moreover, collisional absorption also happens implying the inadvertent impact absorption of nanoemulsion droplets [85]. Upon absorption, nanoemulsion may enter the systemic circulation through the hepatic portal vein or be interfaced into the perforated lymphatic endothelium [86]. Drugs absorbed

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solubility and low bioavailability when administered in the form of nanoemulsion.

3. Summary of selected oral antihypertensive drug-loaded nanoemulsion.

Drug candidate	Oil phase/surfactant/cosurfactant	Method of preparation	Outcomes	Ref.
Red ginger	Coconut oil/Tween 80/PEG 400	Water titration method	Red ginger provides antihypertensive action by inhibiting ACE	[88]
Nisoldipine	Peceol/Cremophor EL/Transcutol HP	Ultrasonication technique	Improved bioavailability and antihypertensive activity	[89]
Nitrendipine	Capmul MCM, Triacetin/Kolliphor ELP/Transcutol HP	Spontaneous emulsification method	Improvement in penetration of drug	[90]
Raspberry ketone	Sefsol 218®/Tween 80/Lauroglycol 90	High energy emulsification technique	Improvement in aqueous solubility and bioavailability	[91]
Eplerenone	Triacetin/Kolliphor EL/PEG 400	Ultrasonication technique	Improved bioavailability of the drug	[92]
Mebudipine	Ethyl oleate/Tween 80/PEG 400	Sonication	Improved bioavailability	[93]
Olmesartan medoxomil	Soyabean oil 700/Sefsol 218/Solutol HS 15	Phase inversion technique	Improved pharmacokinetics and therapeutic efficacy of the drug	[94]
Ramipril	Sefsol 218/Tween 80/Carbitol		The improved bioavailability of the drug	[95]
Candesartan cilexetil	Soyabean oil/Solutol HS-15/Tween 80	Solvent evaporation technique	Improved oral absorption of the drug	[96]

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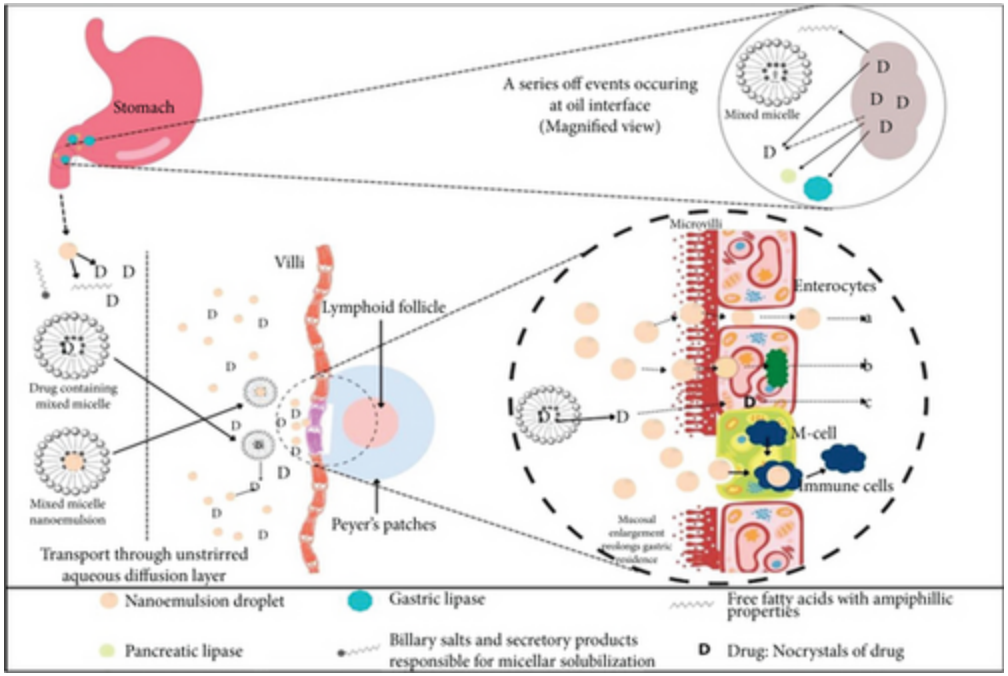

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3.2. Comparative Analysis of Nanoemulsion versus Another Nanostructured Delivery System

This analysis was carried out by studying research publications published between 2016 and 2021 in the Science Direct, Pubmed, Springer, Taylor & Francis, Google Scholar, and EBSCO databases on nanostructured drug delivery systems to deliver antihypertensive drugs for the management of hypertension. Nanoemulsion for hypertension, novel drug delivery system for hypertension, advanced drug delivery system, solid lipid nanoparticles for antihypertensive drugs, polymeric micelles for hypertension, nanosuspension to deliver antihypertensive, self-nanoemulsifying drug delivery system for antihypertensive, and advanced drug delivery system for hypertension were the keywords to study the research publications. Only articles written in

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meantime, the ones that were not relevant were eliminated. According to an exhaustive study of research publications from 2016 to 2021, nanoemulsion has captured a large market segment in comparison to other nanostructured drug delivery systems for delivering antihypertensive drugs. Figure 7 using a pie chart depicted that nanoemulsion (34%) has a presiding role superseded by solid lipid nanoparticles (29%), self-nanoemulsifying drug delivery systems (27%), nanosuspension (7%), and polymeric micelles (1%) in descending order to deliver the antihypertensive drugs.

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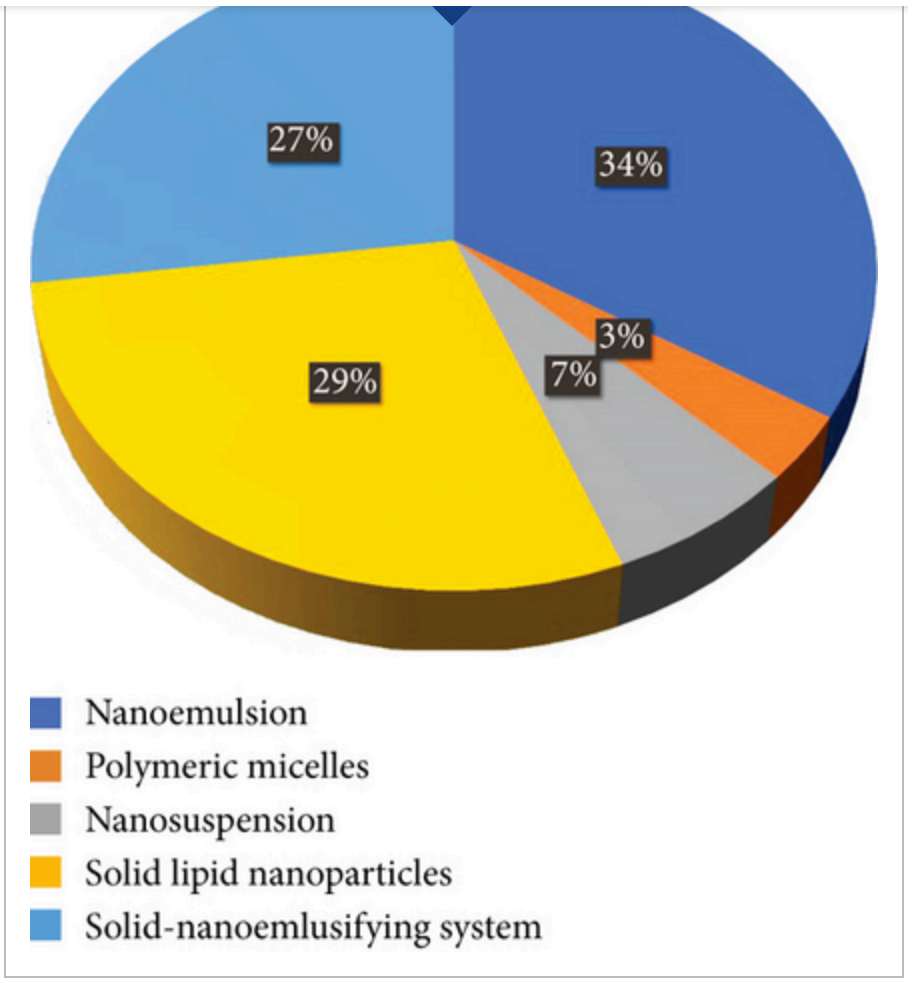

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3.3. Pharmacodynamic Studies of Nanoemulsion Delivering Antihypertensive Drugs

According to a published research by Nada et al., it was found that unilateral ureteral obstruction rats that were given red ginger nanoemulsion had a substantial reduction in systolic blood pressure from 142 ± 1 mmHg to 107 ± 6 mmHg and diastolic

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significant reduction in systolic blood pressure from 187.31 ± 18.02 mmHg to 139.68 ± 13.12 mmHg and diastolic blood pressure from 122.36 ± 12.01 mmHg to 101.84 ± 10.28 mmHg [89]. In a research study by Gorain et al., it was observed that systolic blood pressure (115.71 ± 4.800 mmHg) was reduced after 1 hour of administration of Olmesartan nanoemulsion and was controlled up to 12 hours after administration (120.12 ± 5.724 mmHg). The considerable reduction in blood pressure persisted until the 14th day of treatment (108.96 ± 3.24 mmHg at 1 hour and 114.72 ± 3.74 mmHg at 12 hours after administration) until it reached the normal systolic blood pressure [94]. In a study by Chhabra et al., the uptake of amlodipine besilate from the nanoemulsion formulation was higher in almost every organ, especially in the heart than suspension of amlodipine besilate, confirming the targeting activity of nanoemulsion. Percentage uptake of the drug was found to be 11.8 and 13.6 folds greater in the heart at 0.5 hours and 1.0 hours, respectively, from nanoemulsion of amlodipine besilate than suspension of the drug [98].

3.4. Patents for Antihypertensive Nanoemulsion

A patent is the government's formal right to grant an inventor the sole right to create, sell, or use a product for a specific amount of time [103]. Several patents for nanoemulsion formulations have been issued, demonstrating the widespread acceptance of nanoemulsion formulations [104]. A patent (CN105997873A) was awarded to Zhang Hongli for the preparation of oil-in-water type nanoemulsion containing terazosin as an antihypertensive drug, aiming at prolonging the half-life of the drug, reducing dose frequency, and improving antihypertensive activity of the drug. The patent discloses oil-in-water type nanoemulsion composed of terazosin 1-15%, surfactant 15%-40%, cosurfactant 0-20%, and the remaining amount of distilled water. Ouyang Wuqing et al. granted a patent (CN102698245A) for the preparation of an antihypertensive drug of quinapril hydrochloride and rose oil nanoemulsion aiming at prolonging the half-life of the drug, reducing dose frequency, and improving the therapeutic efficacy of the said drug. The patent discloses oil-in-water type nanoemulsion composed of 1-18% of quinapril hydrochloride, 15-40% of surfactant, 0-20% of cosurfactant, and 1-20% of rose oil along with the balanced quantity of distilled water. More supportive data for granted patents in the field of nanoemulsion for antihypertensive drugs has been summarized in Table 4.

4. Summarized patent approval for nanoemulsion for antihypertensive drug.

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CN105997873A	Terazosin	Oil-in-water type terazosin nanoemulsion antihypertensive drug	Zhang Hongli	The prolonged half-life of the drug, reduced dose frequency, and improved therapeutic efficacy
CN106137958A	Apigenin	A kind of compound apigenin nanoemulsion antihypertensive drug	Zhang Hongli	Improved dissolution and penetration power of drug along with an increased instability
CN106176997A	Atenolol	A kind of compound atenolol nanoemulsion antihypertensive drug	Zhang Hongli	Improved dissolution and penetration power of drug along with an increased instability
CN102698245A	Quinapril	Antihypertensive drug of quinapril hydrochloride and rose oil nanoemulsion	Ouyang Wuqing, Sun Jianhong, Zhang Xiaohua	The prolonged half-life of the drug, reduced dose frequency, and improved therapeutic efficacy
CN102697900A	Spirolactone	Compound spirolactone nanoemulsion drug	Ouyang Wuqing, Sun Jianhong, Cao Tong	Improved dissolution and penetration power of drug along with an increased instability
CN106137961A	Celiprolol	A kind of oil-in-water type celiprolol nanoemulsion antihypertensive drug	Zhang Hongli	Improved stability, prolonged half-life, and enhancement in the therapeutic efficacy of the drug
CN106109410A	Hydralazine	A kind of hydralazine	Zhang Hongli	The prolonged half-life of the drug,

4. Conclusions

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study has been conducted on a variety of drug delivery systems, which concludes that nanoemulsion has a promising future as a delivery system for antihypertensive drugs. In the coming years, nanoemulsion will become a mainstream practice, with all positive results for the betterment of human society and an increase in life expectancy. Nevertheless, many challenges still need to be overcome to establish their safety and efficacy by performing preclinical and clinical studies. Randomized clinical trials should be performed to gain a better understanding of the effects of various crucial parameters such as droplet size, composition, and charge on the absorption, distribution, and metabolism of the antihypertensive drugs loaded in nanoemulsion. Additionally, various advancements in nanoemulsion like multiple nanoemulsions, in situ nanoemulsion, and self-emulsifying nanoemulsion are yet to be explored for cardiovascular and other diseases.

Conflicts of Interest

The authors declare no conflicts of interest.

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Nanotechnology-based strategies for effective delivery of phytoconstituents for the management of rheumatoid arthritis

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Abstract

Rheumatoid arthritis [RA] is a painful disorder that causes inflammation in synovial membrane followed by damage to cartilage and bone. Several therapies are available for its management that include anti-inflammatory and disease-modifying antirheumatic drugs. Due to the severe side effects associated with them, phytotherapy may serve as a promising and beneficial approach for management of rheumatoid arthritis and the therapeutic potential can be attributed to their

ability to target various inflammatory mediators including nitric oxide [NO], cytokines, chemokines, adhesion molecules, nuclear factor kappa-b [NF-kb], lipoxygenase [LOXs] and arachidonic acid [AA]. Further, nanomedicine could serve as a prominent formulation strategy to overcome the challenges associated with phytoconstituents like poor bioavailability, high first pass metabolism and lower stability. Present review focuses on providing an exhaustive account of various phytoconstituents that have significant potential in RA management, their drawbacks, reported novel drug formulations, regulatory aspects involved therein.

Previous Next

Keywords

Autoimmune; Inflammation; Phytoconstituent; Novel drug delivery system; Nanotechnology

1. Introduction

RA is an inflammatory disorder of joints that affects primarily the synovial membrane, which might result in damage to cartilage and bone, ultimately leading to lifelong disability and loss of quality of life [1]. Swelling followed by pain in feet and hands is most common indication of RA [2]. If left untreated the clinical manifestations may include ulnar deviation [3], swan neck deformity [4], and subcutaneous nodules [5]. RA development starts with inflammation of the lining of joint capsule that leads to accumulation of synovial fluid in large amount. This synovial fluid contains enzyme metalloproteinase which further attacks and erodes the cartilage [6].

Rheumatoid factor [RF] and anti-citrullinated protein/peptide antibodies [ACPA] are the most common serum biomarkers of RA [7]. Women are more likely to get affected with the disease suggesting the relation between hormones and RA development. [8]. The pathophysiology of RA is complex and there are several factors that are responsible for RA progression and development. Familial and hereditary genetic associations also contribute to RA development.

Human leukocyte antigen [HLA] genes are considered to be associated with RA [9]. Some common factors that are responsible for this chronic autoimmune disease are listed in Fig. 1. All these factors viz; pro-inflammatory cytokines, pro-inflammatory enzymes, expression of adhesion molecule, matrix metalloproteinases, and synovial fibroblasts hyperproliferation are regulated by transcription factor nuclear factor-kB [10]. The auto-antibodies like immunoglobin- M [IgM], immunoglobin G [IgG] and immunoglobin A [IgA] commonly known as anti- rheumatoid factors also play major role in RA progression [11]. They may cause production of neutrophils, lymphocytes and cytokines by ligation of Fc-c receptors on macrophages leading to inflammation in RA [12,13]. Other factors included smoking and stress, which also contribute to the progression of this disease [14]. The treatment goal is to reduce pain, inflammation and to achieve remission or at least to reduce the activity of disease to provide relief to the patients [15]. Several treatment options are available currently which include non-steroidal ant-inflammatory drugs [NSAIDs] like ibuprofen and acetaminophen and glucocorticoids, Disease modifying anti-rheumatic drugs [DMARD] like methotrexate, sulphasalizine and biological agents like tumor necrosis factor-alpha [TNF-a] blockers and interleukin-1 receptor antagonist [IL-1Ra] [16]. However, all of these are associated with various side effects like renal, hepatic and cardiotoxicity associated with NSAIDs [17], peptic ulcer, osteoporosis and increased rate of infection may occur with use of glucocorticoids [18]. Likewise, use of DMARDS may cause tuberculosis, chronic fungal infections, lymphomas and liver injury. Biological therapy is safer when compared to all these treatments. However, many patients are not able to afford the therapy and stop the treatment after short- term usage because of the high cost and availability in the form of injection [19]. Owing to these limitations, lot of research are exploring herbal resources for promising phytoconstituents for the treatment and management of RA. Natural products are safer and that tends to increase adherence of patients towards therapy. However, these phytoconstituents need to be evaluated in terms of safety and efficacy [20].

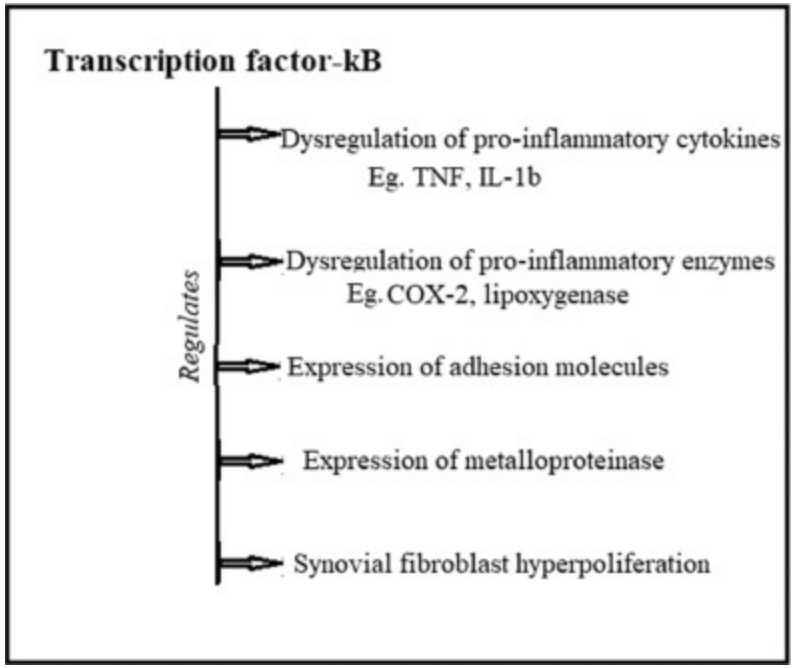

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Fig. 1. Pathophysiology of RA.

2. Significance of phytoconstituents for the treatment of rheumatoid arthritis

Traditional Chinese medicine is being used in China since ancient times for RA treatment and management and is constantly gaining worldwide acceptance. Presently various TCM are being for RA treatment that show good clinical efficacy [21]. Most importantly various phytocompounds extracted from Chinese herbs have shown promising results *in vivo* and *in itro* when evaluated for their potential towards inflammation and arthritis with specific action.

Zheng Qing Feng Tong Ning (ZQFTN) is an example of TCM patented drug that consists of phytoconstituent sinomenine (SIN) and was approved in 2013 by China Food and Drug Administration (CFDA) for RA treatment. Due to its low toxicity and high clinical efficacy for RA treatment, it has now has been accepted by *National Health Insurance Directory* of China for RA

therapeutics [22]. Berberine is derived from TCM. Dai-Zong-Fang (DZF) is a herbal formulation that consist of *Rhizome coptidis* and *Fructus aurantii* Immaturus of which berberine, naringin and hesperidin are the main constituents and is approved for use as first line therapy in Type 2 diabetes [23]. Qingfei Huatan decotion, Huzhang Oral Liquid, Feiyu Granules and Baihe Gujin Pill are the classical prescriptions that can be used for pneumonia, COPD and asthma. *Polygonam Cuspidatum*, Huzhang is the main ingredient of the above prescription that contain phytocompound Resveratrol Resveratrol is the commonly prescribed and used medicine in China and Japan [24]. berberine, nariginin, hesperidin and resveratrol were also reported to have favorable therapeutic efficacy for treating RA. Quercetin is another plant derived constituent under TCM that offers anti-inflammatory, anti-viral, anti-hypertensive and anti- proliferative effects. Several clinical trials are being conducted in China to explore quercetin for its antiviral potential in the current pandemic conditions [25]. Various research reports have reported quercetin to be a potential compound for RA management. Several of Chinese proprietary medicine obtained from TCM formula or bioactive phyto compound like controlled- release Zheng Qing Feng Tong Ning (ZQFTN) Tablets, Tripterygium glycoside tablets, and ottal glucosides of Peony (TGP) capsules are the part of *National Health Insurance Directory* of China and have shown therapeutic effect similar to the chemical drugs like methotrexate [26,27]. These phytoconstituents derived from TCM can benefit RA patients and offer advantage over western chemical drugs like fewer or no side effects, low toxicity, efficacy comparable to western chemical drugs, multiple target and specific action are the most suitable therapeutic approach disease that requires prolonged treatment like RA [27].

Thus, the aim of this review is to cover various phytoconstituents that have been reported to exhibit potential benefits in RA treatment, novel drug delivery systems for their delivery and challenges associated with their design and development. Further various toxicity and regulatory issues associated with phytoconstituents are also highlighted.

3. Phytoconstituents having potential activity in rheumatoid arthritis

Several phytoconstituents isolated from plants have been demonstrated to downregulate the pro-inflammatory signals and thus may have potential for treating RA [28]. Due to limited efficacy and numerous side effects of available therapies, around 60-90% patients depend on complementary and alternative medicines derived from natural plants. Phytoconstituents derived from various natural sources alone or in combination can control arthritis through multiple pathways [29]. Some of the well-studied natural products for RA, there sources and molecular targets are listed in Table1. and are detailed below. The chemical structures of these phytoconstituents are depicted in Fig.2.

Table 1. Phytoconstituents, their IUPAC name, source and molecular target.

Phytoconstituents	IUPAC name	Source	Molecular target
Curcumin	1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6- heptadiene-3,5-dion	Rhizomes of Curcuma longa [tumeric] *Zingiberaceae*	NF-kB, COX-2, 5-LOX, TNF-a, IL-1b, IL-6, IL-8, MMPs, AMs
Resveratrol	3,5,4′-Trihydroxystilbene	Vitis vinifera [red grapes]	NF-kB, COX-2, TNF-a, 5-LOX, AMs
Berberine	16,17-dimethoxy-5,7-dioxa-13-azoniapentacyclo[11.8.0.$0^{2,10}$.$0^{4,8}$.$0^{15,20}$]henicosa-1(13),2,4(8),9,14,16,18,20-octaene	Bark of Berberis vulgaris and berberis aristata, Berberidaceae,	NF-kB, COX-2, TNF-a, IL-1b, IL-6, MMP-9
Thymoquinone	2-isopropyl-5-methyl-1,4- benzaquinone	black Caraway seed of *Nigella sativa* Ranunculaceae	IL-1β, TNFα, COX-2, MMP-13, PG-E2
Celastrol	10-Hydroxy-2,4a,6a,9,12b,14a- hexamethyl-11-oxo- 1,2,3,4,4a,5,6,6a,11,12b,13,14, 14a,14b-tetradecahydro-picene-2-carboxylic acid	*Root and bark of Tripterygium wilfordii,* Celastraceae	MMP-9, COX-2, JAK-1, JAK-3, IKK-β, SYK, MMP-3, JNK and MEK1, TNF-a, IL-6
Hesperidin	3,5,7-trihydroxyflavanone-7-rhamnoglucoside]	Fruit of *Citrus aurantium,* Rutaceae and Lamiaceae	IL-1, IL-6 and TNF-alpha
Gamboic acid	*Z*)-4-[12-hydroxy-8,21,21-trimethyl-5-(3-methylbut-2- enyl)-8-(4-methylpent-3-enyl)- 14,18-dioxo-3,7,20-trioxahexacyclo[15.4.1.$0^{2,15}$.$0^{2,19}$.$0^{4,13}$.$0^{6,11}$]docosa-	Resin from Garcinia hanbaryi Hook, Moraceae	MMP-2, MMP-9, nuclear factor [NF]-κB,

Phytoconstituents	IUPAC name	Source	Molecular target
	4(13),5,9,11,15-pentaen-19-yl]-2-methylbut-2-enoic acid		
Sinomenine	9α,13α,14α -7,8-Didehydro-4- hydroxy-3,7-dimethoxy-17- methylmorphinan-6-one hydrochloride [	Root of *Sinomenium acutum,* Menispermaceae	COX2/PGE2, TNF, INF-*γ*, IL-6, IL-1*β*, and IL-4 MAPK, MMPs, and NF-κB
Epigallocatechin-3-gallate	[(2R,3R)-2-(**3**,4-dihydroxyphenyl)-5,7- dihydroxy-**3**,4-dihydro-2H- chromen-**3**-yl] **3**,4,5-trihydroxybenzoate	*Leaves of Camellia sinensis [green tea]*	cytokines, chemokines, MMPs, aggrecanase, reactive oxygen species [ROS], nitric oxide [NO], COX-2, and PGE2
Triptolide	(1*S*,2*S*,4*S*,5*S*,7*R*,8*S*,9*S*,11*S*,13*S*) -8-hydroxy-1-methyl-7-propan- 2-yl-3,6,10,16-tetraoxaheptacyclo[11.7.0.$0^{2,4}$.0 ^{2,9}.$0^{5,7}$.$0^{9,11}$.$0^{14,18}$]icos-14(18)-en-17-one.	*Tripterygium Wilfordii Hook f.*	IL-6, IN-10, IL-12, Th17 cells, IL-17, tumour necrosis factor [TNF]-α, IL-1β, nuclear factor [NF]-κB, and [COX]-2 and cytokines
Quercetin	3, 3′, 4′, 5, 7-pentahydroxyflvanone.	Camellia Sinensis, Moringa oleifera *Ginkgo biloba, Hypericum perforatum*, Apium graviolens, Brassica oleracea ,*Sambucus canadensis* etc	COX, LOX, TNF-α, IL- 1β, IL-6 α IL-17, and MCP-1
Ferulic acid	4-hydroxy-3-methoxycinnamic acid	*Ferula foetida*	TNF- α, JAK2 levels, and TGF-β

Phytoconstituents	IUPAC name	Source	Molecular target
Emodin	1,8-dihydroxy-3- (hydroxymethyl)anthracene-9,10-dione	Rheum palmatum, Polygonum cuspidatum and Polygonum multiflorum.	IL-1β, NF-κB,TNF- α, interferon-gamma

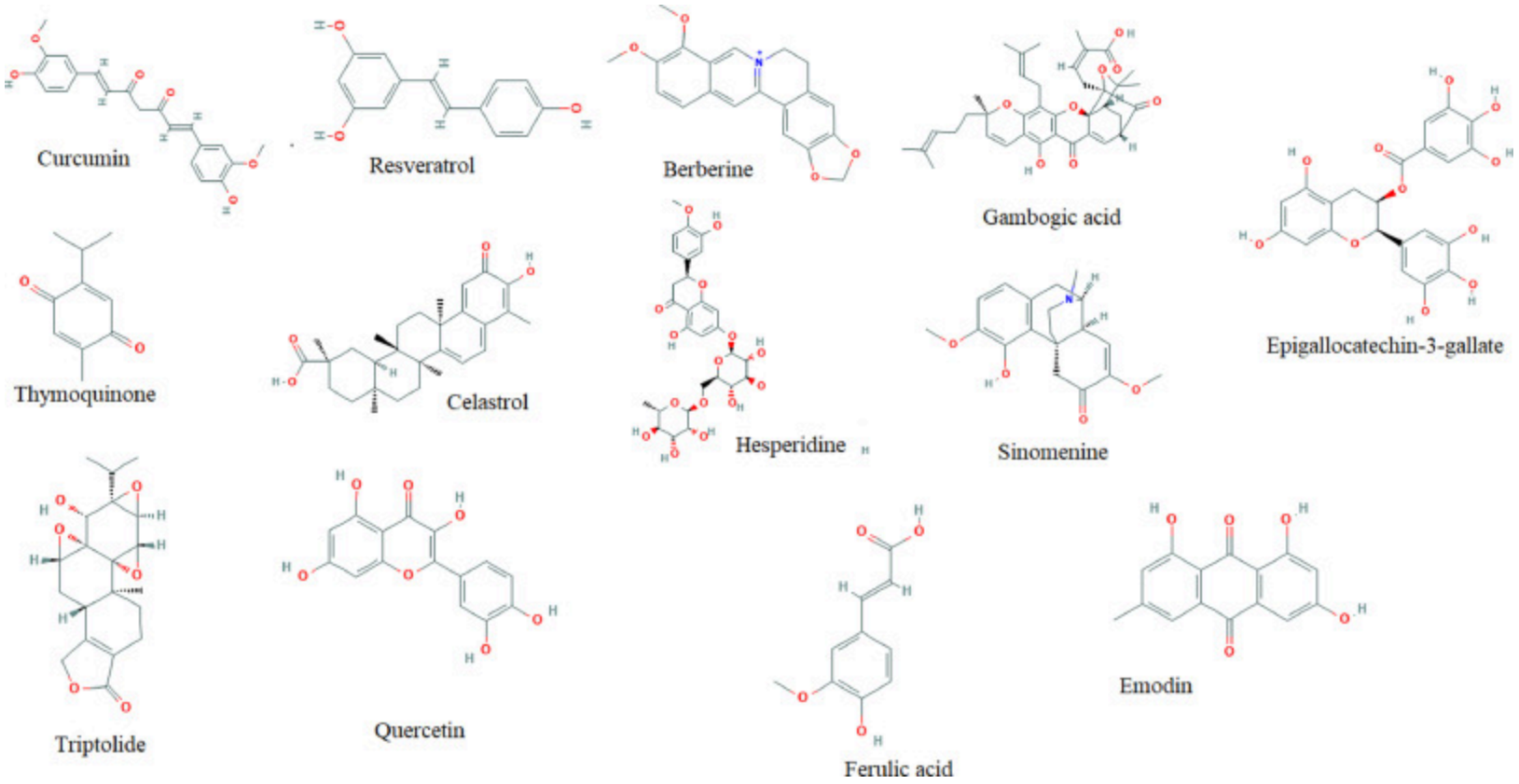

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Fig. 2. Chemical Structure of Phytoconstituents.

3.1. Curcumin

Curcumin, a hydrophobic polyphenol is derived from rhizomes of *Curcuma longa* [30] and is reported to possess remarkable anti-inflammatory activity [31]. Curcumin is also known as diferuloyl methane and is symmetrical in structure (Fig.2) [32]. Curcumin downregulates various factors like nuclear factor kappa B [NF-kB], cyclooxygenase-2 [COX-2], 5- lipoxygenase [5-LOX], TNF-a, interleukin-1b [IL-1b], interleukin-6 [IL-6], interleukin-8 [IL-8] and matrix metalloproteinases [MMPs] and thus seems to be effective bioactive for RA treatment [33], [34], [35], [36], [37]. Several studies have been conducted to demonstrate the involved mechanism and potential of curcumin against arthritis. Wang et al. have evaluated the therapeutic effect of curcumin and pharmacological mechanism. Curcumin was administered orally at daily dose of 200mg/kg and 100mg/kg in collagen- induced arthritic rats. Results demonstrated significant decrease in TNF-α, IL-17, IL-1β and TGF-β level in rat synovium and TNF-α and IL-17 in serum of rats when treated with 200 mg curcumin and methotrexate when compared to control group ($p<0.05$). Further, proportion of apoptotic macrophages was detected using TUNEL (TdT-mediated dUTP nick end labelling) in-situ staining. Only (2.7 ± 0.7%) of apoptotic cells were found in synovium or cartilage of ankle joint in rats. Further in the cartilage edge of the rat joint the TUNEL-positive macrophages were increased to (16.2 ± 3.2%) in 100 mg/kg group and (19.9 ± 4.2%) in 200 mg/kg group when compared to control group of CIA rats ($p<0.05$). The result provided the evidence that curcumin can attenuate joint swelling by downregulating cytokines, inhibiting NF-κB activity, reducing COX-2 level, and promoting macrophage apoptosis [38]. In 2018, Dai et al. investigated the effect of curcumin in Wistar rats. Collagen- induced arthritis [CIA] was developed in rats and afterwards treated with curcumin 200mg/kg daily for 3 weeks. Curcumin was shown to attenuate the mammalian target of rapamycin [mTOR], a new target for RA therapy. mTOR expression was 0.299±0.024 in CIA rats, that decreases to 0.226±0.032 in Curcumin treated group ($P<0.05$). The treatment inhibited the increase level of proinflammatory cytokines like IL-1β (483.06±73.06), TNF-α (426.31±51.01), MMP-1 (219.13±14.23), and MMP-3 (99.12±4.37) to IL-1β (254.02±55.90), TNF-α (248.37±44.42), MMP-1 (182.26±15.40) and MMP-3(84.19±5.09)pg/mL in rat serum and also reduces levels of IL-1β, TNF-α and IL-6 in rat synovium when compared to CIA group with no treatment. ($P<0.05$). Reduction in inflammation and swelling in joints of rats was also observed [39]. Zheng et al. evaluated the potential of curcumin in adjuvant- induced arthritis in rats. Curcumin was shown to be as effective as methotrexate, a DMARDs and the levels of TNF-α and IL-1β were found to decrease in both synovial fluid and blood serum after the oral administration of curcumin oil-in-water nano-emulsion and I.V. injection. The expression of NF-κB in the synovial tissue using immunohistochemical analysis was determined and it was found that in animals treated with CM or MTX, the intensity and positivity were significantly reduced or eliminated. It was also found that Treatment with CM or MTX

significantly reduced the TNF-α and IL-1β levels compared with the model group, with approximately fourfold and threefold decreases observed for TNF-α and IL-1β in synovial fluid, respectively, and approximately twofold for both cytokines in blood serum [40].

3.2. Resveratrol

Resveratrol, a natural polyphenol with a stilbene backbone [Fig.2] [41], was first isolated from roots of *Veratrum grandiflorum O. Loes* but is now reported to be present in various plants that include grapes, berries and peanuts [42]. Resveratrol in various tissues can alter pathways that include extracellular signal-regulated kinases [ERK], mitogen activated protein kinase [MAPK], Activator protein 1 [AP-1] and NF-κB [43]. Yang et al. used bovine type-II collagen [BIIC]- induced arthritis model in Sprague-Dawley rats and IL-1β-stimulated rat synovial cells [RSC-364] i.e. *in vitro* RA model. ROS can promote proinflammatory cytokines expression through JNK- and p38 MAPK signalling pathways. Downregulation in hypoxia-inducible factor-1α (HIF-1α) in IL- 1β-stimulated RSC-364 cells with decrease in level of activated JNK (c-Jun N-terminal kinase) and p38 MAPK (mitogen-activated protein kinase)was observed in in-vitro RA model. Resveratrol was administered orally in a dose of 200 and 400 mg/kg and the arthritis score index on day 42 after BIIC immunization was reduced to 5.36 from 8.37 by 400 mg/kg body weight of resveratrol ($p < 0.01$). Results revealed nearly three-fold decrease in histological score (0.62 from 1.73). Further the changes in serum malonaldehyde (MDA) and superoxide dismutase (SOD) and level of proinflammatory cytokines was reversed with resveratrol ($P<0.01$). Thus indicating preventive role of drug in RA by reducing (reactive oxygen species) ROS accumulation, inflammation, and angiogenesis in the synovial tissue by mitogen-activated protein kinase (MAPK) signalling pathways which are considered to be responsible for managing the production of pro-inflammatory cytokine [44]. Further, in another study by Cheon et al. the effect of resveratrol along with dietary supplementation was evaluated. The severity of arthritis was found to be reduced to 2.7±0.6 from 6.7±0.8. Bone destruction on radiograph was reduced to 2.0 ±0.2, from 3.4 ± 0.3. Significant reduction in inflammation 2.0±0.3 from.3.2±0.2, cartilage damage 1.5±0.3 from 3.2±0.2, pannus formation 1.4±0.3 from 3.0±0.3, erosion; 1.4±0.2 from 3.3±0.3 ($P<0.01$). Safranin-O and tartrate-resistant acid phosphatase (TRAP) staining, in addition to radiographic imaging of the paws showed positive effect in bone erosion as well as cartilage damage in CIA rats ($P<0.01$) [45].

3.3. Berberine (BBR)

Berberine is a bis-benzyl iso-quinoline alkaloid (Fig.2) [46] found in *Berberis vulgaris*. Berberine is also reported to downregulate various factors including NF-kB, COX-2, TNF-a, IL-1b, IL-6 [47]. To prove the efficacy of berberine in RA, Wang etal. administered berberine [75 and 150 mg/kg] in Freund's complete adjuvant [FCA]- induced arthritis rats. In high dose treated group treated group the arthritis index scores were reduced to 7.81 ±0.03. ($P<0.01$) The result showed alteration in the levels of IL-6, IL-10, IL-17 and TGF-b ($P<0.01$) in serum. Further significant decrease in of anti-IL-17 antibody was observed ($P<0.001$). Regulation of balance between Treg and Th17 cells was proposed as probable mechanism for its significant protective effect [48].

In another study by Vita etal. , BBR [1 mg/kg/days] successfully treated CIA rats through T cell suppression that affects B cell activity indirectly. . It was found that only 40 percent mice developed CIA in BBR treated group as compared 90 percent in control group and 80 percent in PBS control group. The authors suggested BBR to be a potential prophylactic supplement [49].

3.4. Thymoquinone (TQ)

Thymoquinone is the major active component of plant *Nigella sativa* was reported to possess anti- inflammatory, analgesic and antioxidant properties [50]. It is a monoterpene molecule (Fig.2). Arjumand etal. investigated the potential of bioactive against RA. TQ, 10 mg/kg of body weight was given intraperitoneally to Freund's Complete Adjuvant (FCA)-induced arthritic rats after the 8th day of induction and the arthritic score was decreased to (2.1 ± 0.56) when compared to arthritic control group (2.7 ± 0.48) ($p<0.01$). Significant decrease in levels of CRP 22.1 ± 1.79 from (32.2 ± 2.09); $p < 0.001$), synovial inflammation 1.5 ± 0.54 from 2.16 ± 0.40, pannus formation 1.1 ± 0.73 from 2.0 ± 0.94 and bone erosion (0.8 ± 0.42 from (1.4 ± 0.51) was seen.. Downregulation of TLR2, TLR4, TNF-α, IL-1, and NFκB expression levels ($P<0.001$) was the mechanism reported by the authors [51]. Further Tekeoglu etal. evaluated the anti-arthritic effect of TQ [2.5 and 5mg/kg] in FCA-induced arthritis model. The initial score of arthritis, radiological, level of TNF-alpha, IL-1beta were 2,28, 1.85, 1.63 pg/mL,14.85 pg/mL and after treatment with Thymoquinone are 2.5 mg/kg 0.71,0.71,0.81, 8.87 pg/mL and for Thymoquinone 5 mg/kg 0.57, 0.57, 1.04, 2.74 respectively ($p<0.05$).Thus Clinical and radiological scores confirmed the suppression of adjuvant-induced arthritis in rats by decreasing the level of TNF- alpha and IL-1beta Clinical and radiological scores confirmed the suppression of adjuvant-induced arthritis in rats by decreasing level of TNF-alpha and IL-1beta [52].

3.5. Celastrol

Celastrol is a chinese traditional medicine obtained from *Trypterugium wilfordii* [53]. Chemically it is a pentacyclic triterpenoid (Fig.2) with antioxidant and anti-inflammatory activities. Yuan et al. evaluated the efficacy of celastrol against RA in adjuvant-induced arthritis murine model. After the onset of the disease, the drug was administered intraperitoneally daily. TNFα and IL-6 levels were reduced by 1.9-fold and 3.1-fold after the Celastrol administration when compared to the group treated with PBS(Phosphate buffer solution). Inflammation in joints of arthritic mice was suppressed with reduction in myeloperoxidase [MPO] and neutrophil elastase [NE] concentrations [54].

Further in another study Astry et al. confirmed the anti-arthritic activity of drug after intraperitoneal administration at daily dose of 1mg/kg bodyweight to adjuvant- induced arthritis rats. In the synovium the average ratio of f IL-17-producing $CD4^+$ T cells to Treg (Th17/ Treg) reduces from 23.7% (vehicle treated control group) to 2.3% (celastrol treated group) and for $CD8^+$ T cells to Treg it reduces from 5.3 to 0.3 ($p<0.05$). The mechanism involved was downregulation of IL-1β, TNF and NF-k β [55].

3.6. Hesperidin (HSN)

Hesperidin, a disaccharide derivative (Fig.2) is a flavonoid present mainly in citrus fruits and is known for wide range of pharmacological actions. Its beneficial effect in RA is also well documented. In a recent study of Qi et al. assess HSN in FCA-induced arthritis in mice using intraperitoneal injection (20 mg/kg/day). Study on animals confirmed the improvement of synovial inflammation and reduction in cartilage destruction by modulating MMP synthesis [56].

Further Umar et al. investigated the therapeutic potential in adjuvant rat arthritis model. HSN was given at oral dose of 80,160 mg/kg significantly reduced the secondary paw swelling. The arthritic index was reduced to 1.85 ± 0.38 from 2.42 ± 0.20. Also hesperidin at a dose of 160mg show significant reduction in neutrophil activation and infiltration ($p<0.05$). Articular elastase, nitric oxide and lipid peroxidation was found to be increased in joints of arthritic rats while reduced glutathione level was increased from 0.610 ± 0.09 to 0.735± 0.08, superoxide dismutase activity from 5.92 ± 0.29 to 7.98 ± 0.28 and catalase activity from 9.45±0.27 to 13.52±0.37 when treated with hesperidin [57].

3.7. Gambogic acid

Gambogic acid is a xanthonoid (Fig.2) which is derived from dry resin secreted by *Garcinia hanburyi* and *Garcinia Morella* [58]. Wang etal. reported its potential anti-arthritic effect in collagen-induced arthritic mice. The arthritic scores were reduced to around 4.8 from 1.2 after 28 days of administration ($p<0.01$).The tumor necrosis factor (TNF)-α expression were reduced from 225pg/mg to 149pg/mg, interleukin (IL)-1β (from 55 to 24 pg/mg), IL-6 (98 to 39pg/mg) and IL-18(65 to 28pg/mg) concentrations Also Caspase-3 and caspase-9, matrix metalloproteinases (MMP)-2, MMP-9, nuclear factor (NF)-κB and phosphorylated-p38 protein expression were found to be suppressed when compared to RA model ($p<0.01$). Interestingly, increase in tissue inhibitors of matrix metalloproteases-1 (TIMP-1) protein was also [59].

Wu etal, 2017 elucidated the effect of gambogic acid in rheumatoid arthritis rats at dose of 1mg/kg/day, 5mg/kg/day and 10mg/kg/day. The results demonstrated the significant inhibition of degree of right foot swelling, increased thresholds to pain and improved clinical arthritic scores in all the three group with highest in 10mg group ($p<0.01$). Interleukin (IL)-1β and IL-6 levels were found to be decreased (9.0 to 3.9pg/mg) and (6.0 to 2.8) respectively in 10mg group along with favorable expression of phosphorylated (p)-Akt serine/threonine kinase (Akt) (increased from 35 to 90%), p-mammalian target protein of rapamycin (mTOR) (45 to 86%) in comparison to RA model ($p<0.01$) [60]

3.8. Sinomenine (SIN)

Sinomenine, a tetracyclic alkaloid (Fig.2) is an active ingredient of *Sinomenium acutum,* known for its strong anti-inflammatory properties [61]. SIN efficacy in arthritis treatment was investigated by Liu etal. in CIA rat model. Further, its cytotoxic potential in SIN-RAW264.7 cells was also investigated. The cells were found to be stable after 24-h incubation with 0.1– 50μg/mL SIN whereas the highest concentration resulted in reduction in cell viability. Intragastric administration of SIN was done at dose of 50 and 100 mg/kg. The arthritic score was reduced to 5.9 and 9.0 in 50 and 100 mg group respectively from 13 after 42 days treatment. Inhibition of IL- 6, granulocyte-macrophage colony-stimulating factor [GM-CSF], interlekin-12 p 40 [IL-12 p40], IL-1α, TNF-α, IL-1β, chemokine [CXCL1], Eotaxin-2, IL-10, macrophage colony stimulating factor [M-CSF], RANTES, and MCP-1 secretion was reported accounting for its anti-arthritic effect [62].

Its anti-arthritis properties were also evaluated by Tong etal. in CIA rats. SIN [60, 120 mg/kg] was administered orally, Significant reduction in arthritic score (4.38 ± 0.47 over 8.25 ± 0.73; $P < 0.01$) and paw swelling (1.07 ± 0.14 mL versus 1.99 ±

0.08 mL; P < 0.01) was seen in group receiving 120mg of drug and the results proved that SIN regulated IL- 6, MMP- 2 and MMP- 9 secretion [63].

3.9. Epigallocatechin-3-gallate (EGCG)

Epigallocatechin 3-gallate (Fig.2) is the main active ingredient of green tea i. e. *Camellia sinensis* and it is one of the widely studied molecule for its potential with several health benefits [64]. Its efficacy in arthritis has been well- proven in various studies. EGCG provides relief to RA patients by modulating the expression of cytokines, chemokines, MMPs, aggrecanase, reactive oxygen species [ROS], nitric oxide [NO], COX-2, and prostaglandin-E2 [PGE2] [65]. Leichsenring et al. used *Dark Agouti* rats with pristane-induced arthritis [PIA] for screening an active constituent of green tea. The late application of EGCG does not results in any change in inflammation, however the early oral administration the arthritic scores on 50th day were reduced to 1.9 ± 0.6 from 8.1 ± 2.6 suggesting that only early oral administration can relive the arthritic symptoms via inhibition of MPO [66].

3.10. Triptolide (TPL)

Triptolide is an active diterpenoid triepoxide (Fig.2) obtained from plant *Tripterygium Wilfordii Hook f.* [TWHF]. Numerous studies have been conducted to show its potential in RA. Triptolide is reported to have remedial effect in inflammatory and autoimmune diseases like RA [67]. Triptolide is reported to act by regulating T cell proliferation, IL-6, IL-10, IL-12, T-helper-1[Th17] cells, IL-17, TNF-α, IL-1β, NF-κB, and COX-2 and cytokines expression [68]. Wang et al. identified the effect of triptolide in TNF transgenic mice with RA. Mice were divided into five groups: the control group, low-dose group [3.3 μg/kg/d TPL], middle-dose group [10 μg/kg/d TPL], high-dose group [33 μg/kg/d TPL] and methotrexate group [0.1 mg/kg/d MTX]. Drug was administered with frequency of five days a week for six weeks. When compared to other groups, the high-dose group of TPL was found to have profound decrease in level of IL-1α, IL-1β, and TNF-α. Further the osteoclast precursor apoptosis rate and T-lymphocytes were higher in middle and high- dose TPL group when compared to others [69] . *In vitro* and *in vivo* assay studies have also proven the anti-angiogenic effect of triptolide in CIA- induced arthritis rats by Kong et al. [70] TPL at dose of 11-45μg/kg/day reduces arthritic score and arthritic incidence ($p<0.05$). The expression of factors that includes TNF-α, IL-17, VEGF, VEGFR, Ang-1, Ang-2 and Tie2 were reduced. Another study by Liu et al. proved that triptolide at dose of 8,16 and 32μg/kg can significantly reduce the arthritic symptoms ($p<0.05$). The arthritic index on day 22 was reduced

from 12 to 5 and arthritic incidence lowered to 35% at oral dose of 32μg/kg. Triptolide also reduces the expression of RANKL ($p<0.001$), RANK ($p<0.05$) and ratio of RANKL to OPG (osteoprotegerin) in the sera and inflamed joints ($p<0.1$) [71].

3.11. Quercetin

Quercetin is a naturally occurring flavanoid (Fig.2) found in more than twenty medicinal plants such as *Camellia Sinensis*, *Moringa oleifera*, *Ginkgo biloba*, *Hypericum perforatum*, *Apium graviolens*, *Brassica oleracea* and *Sambucus canadensis* [72]. Quercetin is reported to inhibit TNF-α, IL-1α, inflammation-producing enzymes COX and LOX [73]. Yuan et al. have documented the efficacy of quercetin in RA by inhibition of neutrophil activities in arthritis- induced mice and have proposed this new mechanism for protective effect of quercetin for RA. The arthritic score at 33 day were 2.1 over 4.0 when compared to RA group ($p<0.01$) . The levels of IFN -γ (1.8 to 0.6 pg/mL), IL -6 (6.8 to 5.0pg/mL), TNF (9.0 to 5.9) were also decreased significantly in comparison to RA group along with decrease in expression of MPO (myeloperoxidase) and NE (neutrophil elastase) ($p<0.001$) [74].

In another study, Haleragrahara et al. evaluated the potential quercetin in RA in CIA- induced rats and compared it with methotrexate and combination of quercetin and methotrexate. The study showed that quercetin when administered as monotherapy, alleviated arthritis symptoms more significantly and offered greater protection than methotrexate and quercetin combination. The proposed mechanism was the regulation of factors NF-α, IL-1β, IL-17, and monocyte chemoattractant protein-1[MCP-1]. This study suggested quercetin to be a non-toxic monotherapeutic agent for RA treatment [75].

3.12. Ferulic acid (FA)

Ferulic acid is a phenolic acid (Fig.2). It was first isolated from *Ferula foetida* in 1983. It is most abundantly present in cereal grains [76,77]. Zhu et al. investigated the therapeutic potential of ferulic acid and its possible mechanism of action for RA treatment. In RA, proinflammatory cytokines trigger the activation of Janus kinase-signal transducer and activator of transcription [JAK/STAT] pathway leading to its progression. In this study, Janus kinase 2 [JAK2] inhibition with a docking score of - 6.7 was observed. FA at dose of 25 and 50 mg/kg reduced primary [volume of paw edema] and secondary lesions in FCA-induced arthritis rats. Arthritic scores were reduced to 1.6±0.2 in comparison to model control group 5.8±0.37

($p<0.05$).Changes in biochemical parameters and inflammatory markers such as C-reactive protein [CRP] and rheumatoid factor [RF] were also found to be reversed with decrease in secretion of TNF- α, JAK2 levels, and TGF-β [78].

3.13. Emodin

This natural anthraquinone derivative (Fig.2) is obtained from the Chinese plants such as *Rheum palmatum, Polygonum cuspidatum* and *Polygonum multiflorum*. It is reported to inhibit the expression of NF- κB and production of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α [79]. Hwang et al. have elucidated the mechanism of emodin for arthritis in CIA mice. The result demonstrated the inhibition of the nuclear translocation and DNA binding of NF-κB subunit that further leads to suppression of chemokine production and MMP expression. It was found from the studies that emodin was able to reduce the serum concentration of IL-1β and TNF-α from 120 pg/mL to 20pg/mL and 178pg/mL to 85pg/mL respectively confirming the reduction in inflammation and pain. The number of osteoclast cells responsible for bone degeneration were also reduced by almost 30 folds [80]. Also, Zhu et al. investigated emodin efficacy for RA in murine adjuvant-induced arthritis [AA] model of RA *in vivo* and on neutrophil apoptosis and NETosis *in vitro.* Result showed marked inhibition of neutrophil infiltration and proinflammatory cytokines IL-6, interferon-gamma and TNF-α release in addition to reduction in NETosis in neutrophil. This study further confirms emodin to be a promising phytoconstituent for RA treatment [81].

3.13.1. Other phytoconstituents

Ehretia laevis (E. laevis) is a medicinal plant of extremely high value contains several phytoconstituents like botulin, betulinic acid, lupeol, β-sitosterol, α-amyrin, bauerenol, β-amyrin, lewisone, phytol, pthalic acid etc in its different parts. Yende et al. performed in-silico evaluation to study the effect of E. laevis Phytoconstituents targeting TNF-α. The result demonstrated lupeol and α-amyrin is more effective as compare to the standard drug thalidomide and suggested that these phytoconstituents may provide successful results during preclinical and clinical studies for management of RA [82].

Linalyl Acetate (LA) is the active constituent of *Lavandula angustifolia* Mill (Lavender) oil. LA is reported to possess analgesic, anti-diabetic, antithrombotic and ant-inflammatory activity and it efficacy for RA is investigated by Seo et al. in CIA induced rats. The RA induced rats were then exposed to nicotine to produce more detrimental effects and it was observed that all the alteration including muscle wasting, increased IGF level caused by nicotine was reversed by LA during the experiment and thus LA can serve as a potential phytocompound for RA management [83].

4. Clinical studies

The positive results of phytoconstituents in experimental models of arthritis led to the further evaluation in humans. Therefore, clinical trials have been conducted with several natural active constituents to confirm their efficacy and safety.

Chandran et al. evaluated anti-arthritic property of curcumin [500mg capsule] in forty- five patients diagnosed with RA. Reduction in tenderness and swelling of joint scores was observed in this 8 week treatment, showing great improvement. Overall the improvement was highest for curcumin. Diminution in Disease Activity Score (DAS) (from 6.40 0.73 to 3.55 0.73) ($p<0.05$) . American College of Rheumatology (ACR) scores were 20, 50 and 70 for curcumin, diclofenac and their combination respectively. No adverse effect was reported thus making the drug safe for use in patients in this randomized pilot study [84].

In a randomized, double-blind, placebo-controlled, three-arm, parallel-group study conducted by Amalraj et al., two different doses 250 and 500mg twice daily for ninety days were evaluated and compared in the study. The change in DAS28 (Disease Activity Score 28),VAS (visual analog scale)scores and American College of Rheumatology (ACR) were 66%, 72%, 70% for500mg dose and 53, 62 and 75% for 250mg dose of curcumin respectively. Significant improvement in RA scores were noted and both doses were found to be well- tolerated and safe [85].

Similarly resveratrol (1 gm) also showed decrease in DAS and ACR scores with reduction in swelling and tenderness in randomized controlled clinical trial of 100 RA patients. Significant decrease in serum level of certain biochemical markers like C-reactive protein (2.1 ± 0.4 over 2.6 ± 0.7mg/dl), erythrocyte sedimentation rate (23.5 ± 9.7 over 41.7 ± 20.4 mm/h), undercarboxylated osteocalcin (2.5 ± 0.5 over 4.2 ± 0.6 ng/mL), matrix metalloproteinase-3 (127.4 ± 18.6 over 195.2 ± 32.4 ng/mL), tumor necrosis factor alpha (18.3 ± 6.2 over 29.7 ± 11.8 pg/mL) and interleukin-6 (23.5 ± 7.1 over 51.2 ± 22.1 pg/mL)was also observed when compared to control group ($p<0.001$) in RA patients receiving resveratrol [86].

In another study by Liu et al., a randomized, controlled trial was conducted in forty-nine patients diagnosed with RA to evaluate the efficacy of sinomenine. The drug was also compared with methotrexate. Rheumatologist determined the clinical indices affecting 28 joint disease activity score-28 [DAS 28] before and after the treatment with SIN for 12 weeks. The scores were reduced to 3.28 from 4.19 for 25 RA patients treated with SIN [$P<0.01$]. Approximately equal remission rate was observed with sinomenine and methotrexate [87].

Although these phytoconstituents offer several benefits in RA treatment, long- term clinical studies are still not available to prove their long- term efficacy and safety. Studies involving large number of human subjects for longer duration is still required. Studies with large number of human subjects for longer duration are still required to assure the market availability of safe and effective formulation consisting of phytoconstituents.

5. Drawbacks of phytoconstituents

Despite the potential benefits of above listed phytoconstituents, the major setback associated with then is their low oral bioavailability and high first pass metabolism. The polyphenols possess only 2-20% of bioavailability [88]. The oral bioavailability of curcumin is reported to be 0.47%, has limited tissue distribution and is metabolized rapidly by liver and intestine into curcumin glucuronide or curcumin sulphate [89]. Berberine's oral bioavailability is 0.68% due to poor intestinal absorption and P-glycoprotein efflux [90]. Resveratrol has oral bioavailability of less than 1% due to its extensive metabolism by liver and intestine and the major metabolites are glucuronides and sulfates [91]. Celastrol is also reported to have bioavailability of 3.14% [92]. Sinomenine's oral bioavailability is reported to be 30.46% with higher elimination rate when determined after administration of sinomenine tablets in beagle dogs [93]. It is reported that only 20% of the administered dose of quercetin reaches blood plasma that reduces its bioavailability. Quercetin is detected in plasma as glucuronide, sulphate, and unconjugated form [94]. Emodin also after oral administration undergoes phase II metabolism resulting in its conversion to rhein leading to poor absorption. Increasing the dose does not impart any beneficial change in its bioavailability [95]. Therefore, almost all the phytoconstituents that have potential for RA treatment possess low bioavailability and high metabolism.

6. Toxicity and regulatory concerns associated with phytoconstituents

Various preclinical, clinical, and epidemiological research documents the efficacy of phytoconstituents in treating various inflammatory diseases owing to their antioxidant and anti- inflammatory properties [96]. In current scenario the population is highly exposed to products containing phytoconstituents. So, it is very essential to identify the risks associated with the use of herbal drugs and in this regard, the safety of these products has become an issue of great public health importance [97]. There are a lot of challenges which must be confronted while implementing regulations in the field of phyto and nutraceutical formulations. Challenges often encountered and common to many countries are those related to regulatory

status, assessment of safety and efficacy, quality control, safety monitoring and inadequate knowledge of dynamics and kinetics within national drug regulatory authorities [98].

The definition and categorization of phytoconstituents vary from one country to another. Depending on the regulations applying to foods and medicines, a single phytoconstituent may be categorized as a food, a functional food, a dietary supplement, or a medicine in different countries. This introduces serious difficulty in the definition of the concept of phytoconstituents and products containing phytoconstituents for the purpose of national drug regulation while at the same time also confusing patients and consumers. Additional major challenge in many countries is the fact that regulatory information on herbal medicines is often not shared between regulatory authorities and safety monitoring or pharmacovigilance centers [98].

Lack of suitable quality control, improper labelling and absence of patient information leaflet further affects the safety of the formulation containing phytoconstituents [99]. In developing nation like India with little or no restrain, the nonregistered and poorly regulated products containing phytoconstituents are sold openly in the market. In addition, the excessive and unrestrained use of these products is due to the popular misconception among individuals that these products are not harmful and offers no side effects. Phytomedicines in most of the countries without any compulsory protection and safety toxicological assessment enters to the market and in most of the cases these products are constantly made available to consumers without a prescription [99].

There is no doubt that the increasing cases of toxic effects associated with the use phytopharmaceuticals and nutraceuticals in recent times, is necessitating the need to ensure thorough toxicity assessment alongside active pharmacovigilance on these products to promote their safe use and protect public health [100]. Therefore, the standards for regulation of these phytoconstituent based products must be set up globally and relevant and appropriate measures must be taken by regulatory authorities of different countries to assure safety of these medicines and protect the health of public. Further sufficient knowledge must be shared between the authorities, physician and the patients about the use and safety of these products so that an atmosphere of trust can be developed and these phytoconstituents that have tremendous potential can be utilized more effectively.

7. Nanotechnology-based drug delivery systems

Phytoconstituents have wide potential for arthritis treatment but the limitations like poor water solubility, stability, low bioavailability, and extensive transformation due to first pass metabolism lead to the reduction in therapeutic efficacy of these drugs [101,102]. The physiochemical properties of drug and the vehicle plays important role in absorption of drug. These factors may limit the site-specific action and permeation through skin due to presence of various metabolic enzymes and stratum corneum, the major barrier considered for permeation of any drug [103]. Moreover, the need of increase in dose due to low bioavailability and absorption can cause unwanted toxicity [104].

Nanotechnology- based drug delivery systems [NDDS] utilize principles of nanotechnology to deliver the drugs passively or actively to the targeted tissues, cells, or subcellular domains. This drug delivery system can passively or actively target the abnormal vasculature and impaired lymphatic drainage which prevails in disease like arthritis characterized by inflammation and swelling. This facilitates the penetration and accumulation of nano-sized drug delivery system within the inflammatory microenvironments [105]. Nanocarriers can also be utilized for active targeting of molecules to the targeted cells by binding with appropriate ligands at the surface. Nano- based drug delivery systems can overcome the barriers associated with active targeting which includes, the reticuloendothelial system, angiogenesis, and lack of specificity [106,107]. These barriers lead to short circulation time of medicine, reduced bioavailability to the target site and drug loss and finally expression of side effects. NDDS are ideal for RA treatment as they meet following requirements: [a] suitable size [ranging from 10 to 100 nm] and surface charge [neutral or anionic]; [b] PEGylation can be done to prolong the circulation time; [c] attachment of specific ligands for active targeting [108]. Further nanotechnology- based drug delivery systems like microsphere, lipid nanoparticles, liposomes, nano-emulsion and microemulsion are being developed (Fig.3).

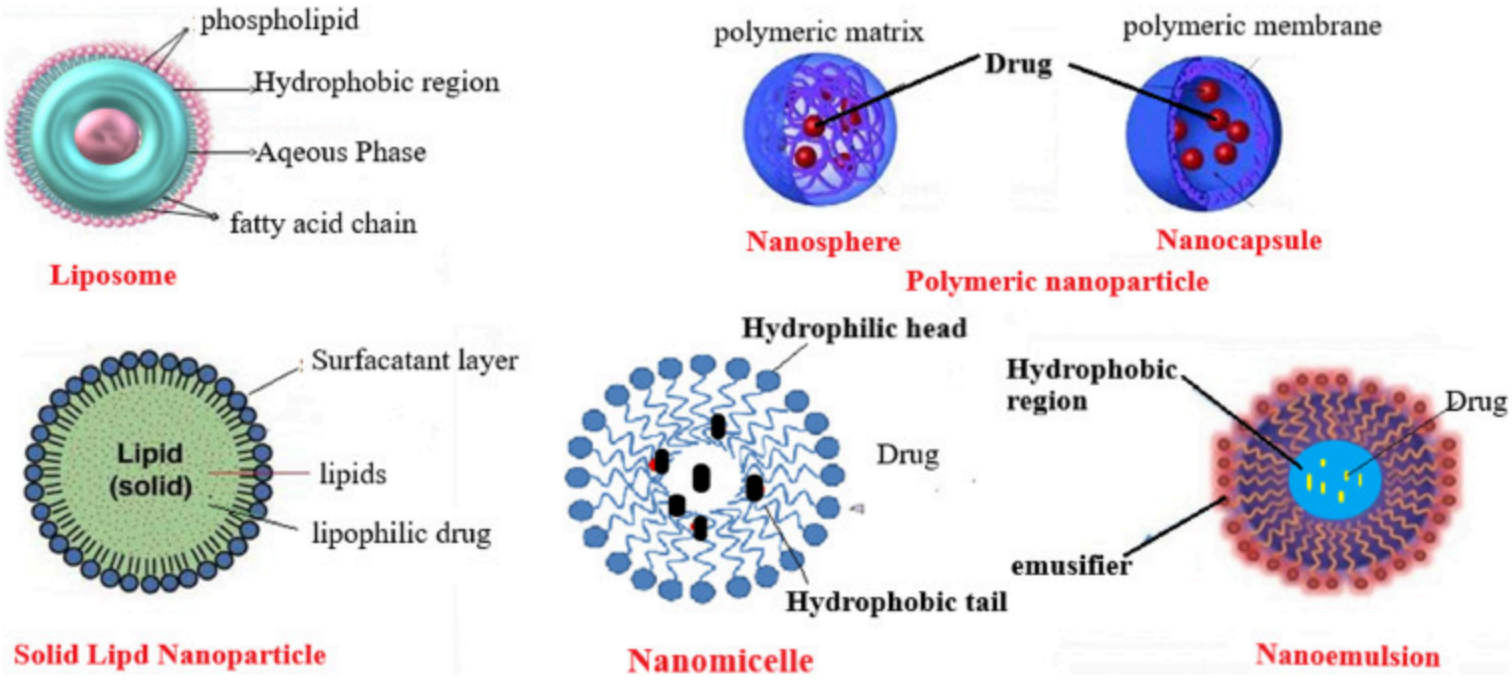

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Fig. 3. Different type of novel drug delivery system.

These systems are modified to offer good and efficient drug delivery strategies in comparison to conventional drug delivery system. They offer several advantages over conventional systems like controlled delivery of both hydrophobic and hydrophilic drugs, high drug- loading capacity, fewer side effects, better stability for systemic and topical drug delivery [109]. Due to the decreased size and increase surface area this system can enter the cell easily and interact with biomolecules. Thus, improved absorption and stability is achieved through nano-system in comparison to conventional systems. Moreover, encapsulated drug can be protected from in-vivo degradation and first pass metabolism. Another important aspect of nano-system is the targeting ability, leading to the increased concentration of drug at target site, improved efficacy and reduced side effects [110].

Nano-system can deliver the drugs to the targeted tissues, cells and subcellular domains either passively or actively [111]. The nano size, surface characteristic and morphology plays vital role in biodistribution of nano-formulations. Various processes like adsorption, ligand receptor attachment, covalent coupling, and inter-nalization will facilitate the uptake of nanocarrier from systemic circulation. In passive targeting abnormal vasculature and impaired lymphatic drainage facilitate the penetration of nano-formulations into the inflammatory microenvironment. Increased angiogenesis results in increase permeability of inflamed endothelial cells as a result of which nano-formulations within the size range of 100nm can permeate passively. On the other side for active targeting the surface of nano-system is modified using suitable ligand that may result in specific binding with the targeted cells. For active targeting nano formulation must possess the following properties-1. Size range from 1-100nm, surface charge (anionic or neutral) 2. PEGylation to increase the residence time 3. Modification using specific ligands [112].

In RA angiogenesis and inflammation are the two vital characteristics. In angiogenesis various relevant mediators like pro-inflammatory cytokines, growth factors, chemo-kines, cell adhesion molecules, and proteases are prominent [113]. Macrophages are also abundantly present in the inflamed synovial membrane. Various receptors like CD44, CD64, scavenger receptor class A, folate receptor-beta (FR-β), vasoactive intestinal peptide (VIP) receptor, toll-like receptors, transforming growth factor-beta receptors, etc. were over-regulated on activated macrophages. Selective delivery to these macrophages can be attained by binding to the suitable receptor that will stop their complicated relation with other cells resulting in delay in RA progression [112].

Table2 covers the various novel nano-formulations and their applications.

Table 2. Developed nano-formulations consisting of phytoconstituents for RA management.

Formulations	Active ingredient	Method of preparation	Average Particle size	Entrapment efficiency	Application of formulation	Reference
Polymeric nanoparticle	Triptolide	Ultracentrifugation	98.0nm	48.6%	Reduction in toxicity	[116]

Formulations	Active ingredient	Method of preparation	Average Particle size	Entrapment efficiency	Application of formulation	Reference
Polymeric nanoparticle	Triptolide	Ultracentrifugation	79.0nm	48.6%	Decrease in toxic effects of triptolide	[117]
Thermosenstive Liposomes	Sinomenine hydrochloride	pH gradient method	116.3nm	90.0%	Reduction in adverse effect, controlled drug release	[119]
Nanoemulsion	Curcumin	high-pressure homogenization	150.0nm	90.0%	Improved drug stability, increased drug absorption	[124]
Nanoemulsion	Curcumin	spontaneous emulsification method	41.1nm	42.9%	Enhanced permeation of curcumin through nano-emulsion gel	[125
Nanoemulsion	Quercetin	spontaneous emulsification technique	136.8nm	94.7%	improved physicochemical stability, acceptable mechanical properties and enhanced skin permeability	[126]
Lipid core nanocapsule	Resveratrol and curcumin	interfacial deposition of preformed polymer	200.0nm	-	Reduced toxicity, improved stability and bioavailability	[129]
Solid lipid nanoparticle	Curcumin	Microemulsification technique	134.6 nm	81.9%	Increased surface area led to increased bioadhesion and thus increased and prolonged cellular uptake, better bioavailability	[131]

7.1. Polymer nanoparticles

Polymeric nanoparticles have size range of 1 to 1000nm comprising active ingredient either entrapped within or adsorbed into the surface of the polymeric core [114]. These are biodegradable, biocompatible and have great potential for target

delivery of drugs [115].

In another study by Zheng et al., nanoparticles consisting TP using poly-γ-glutamic acid- grafted di-tert-butyl L-aspartate hydrochloride [PAT] with average diameter of 79±18 nm, a narrow polydispersity index [0.18], a strong zeta potential [-32 mV] and a high drug encapsulation efficiency [EE1=48.6%] and loading capacity [EE2=19.2%] were formed. *In vivo* study showed increased survival rate of mice and reduced side effects on tumor necrosis factor α transgenic mice, compared to TP. The developed novel nanoparticles were successfully developed with reduced toxicity and increased efficacy .The results clearly indicated the reduction of toxicity of triptolide when encapsulated into a nanoparticulate system, as it was found that all the mice died on the second day when the mice were injected with 2 mg/kg of free TP, and all mice that were injected with 1 mg/kg TP died on day 7. However, the livability was 70% when mice were injected with 0.5 mg/kg of TP. These results indicated that PPT remarkably protected mice from toxicity injury of TP. It was also found that there was no significant decrease in WBCs and RBCs in groups receiving drug in nanoparticulate drug delivery system while on the other hand, a great reduction ($P<0.01$) could be detected in free TP-treated group. Serum analysis indicated that only free TP group induced damage to the kidney (blood urea nitrogen and creatinine) and liver (serum alanine aminotransferase and aspartate aminotransferase} [116].

Zhang *et a.l*,2016 developed triptolide [TP]- loaded nano- drug carrier system [γ-PGA-lPAE-TP [PPT]] with average diameter of 98 ±15 nm, polydispersity index =0.18. the entrapment efficiency was 48.6% with a controlled release manner. HE staining and Tunnel assay results for free TP and TPP was compared, reduced toxicity was observed when PPT was given *in vivo* while TP when administered induced great damage to the kidney, spleen, and liver suggesting PPT to be a promising drug delivery system for RA treatment [117].

7.2. Liposomes

Liposome are spherical- shaped vesicular system consisting of one or more phospholipid bilayer. They are also biodegradable, biocompatible, less toxic and can be used suitably as a carrier for delivery of active constituents at target site [118]. Shen et al. prepared sinomenine hydrochloride- loaded thermosenstive liposomes combined with microwave hyperthermia [SIN- TSL] using pH gradient method. The developed SIN-TSL were having mean particle size of around 100nm, high entrapment and loading efficiency. Drug release was faster at 43°C than the one at 37°C indicating the thermosenstive

behaviour. The system prevents the leakage of drug in the blood and releases completely at RA site under the stimulation of microwave hyperthermia. Further both *in vivo* and *in vitro* results showed superior antiarthritic effect of SIN-TSL combined with microwave hyperthermia [119].

In another study Chen et al. formulated liposomal hydrogel patch loaded with triptolide [TP-LHP]. Here, micro-needle array was used to deliver TP-LHP to promote transdermal absorption in a CIA rat model. Result showed the mitigation of joint swelling and suppression of factors like fetal liver kinase-1, fetal liver tyrosine kinase-4 and hypoxia-inducible factor-1α expression in synovium including reduction in IL-1β and IL-6 levels in serum. It was found from the pharmacokinetic studies that the $t_{1/2}$, T_{max} and AUC was significantly increased from 0.595± 0.321h to 10.381 ± 3.41, 0.982± 0.282 h to 6.59± 0.352 h and 773.63± 68.9 ngh/mL to 4038.45 ± 72.5 ng h/mL respectively when intragastric administration was compared with transdermal delivery of drug. This results clearly demonstrated the sustained release and a longer half-life in plasma as well as decreased hepatic and digestive tract toxicity in comparison to free triptolide [120].

7.3. Microemulsions

Microemulsions are monophasic, thermodynamically stable and clear dispersions consisting of oil, water, surfactant, and a cosurfactant. Microemulsions are effective drug delivery vehicles and can also be used for transdermal delivery of specific active ingredients [121]. Youjun et al. developed sinomenine microemusion- based hydrogel [SMBH] for RA management and evaluated its therapeutic effects on adjuvant- induced arthritis in rats. The mean diameter of the prepared microemusion was 32nm. After 14 days of application of SMBH, the arthritis- induced rats exhibited alleviated paw swelling, decrease in polyarthritis index and reduction in IL-1, TNF-α, PGE2 significantly when compared to sinomenine gel [122].

7.4. Nanoemulsions

Nanoemulsion is a dispersed system consisting of two immiscible liquid phases either oil phase dispersed in aqueous phase or aqueous phase dispersed in oil phase forming droplets of oil or water of nanometric sizes. These systems are stabilized using suitable surfactant [123]. Zheng et al. developed nanoemulsion of curcumin [CM-Ns] to overcome the low oral bioavailability of drug using high pressure homogenizing method for effective management of RA. The prepared CM-Ns have average diameter of 150nm and polydispersity index of 0.23. Further similar decrease in TNF-α and IL-1β in both synovial fluid and blood serum were observed from oral and IV administration [124].

In another study Naz et al. evaluated the potential of nanoemulsion gel- loaded with curcumin for topical delivery. The nanoemulsion was prepared using spontaneous emulsification method using oil [Labrafac PG/glyceryl triacetate], surfactant: cosurfactant [Smix] [tween 80/polyethylene glycol [PEG] 400] and water. The optimized formulation with flux of 117.04±2.32 µg/cm2 /h, droplet size of =41.13±3.34 nm and zeta potential =-33.1±1.45 mV was and incorporated into gel using carbopol-980 [1% w/v]. CLSM study confirmed the maximum deposition of CR up to a depth of 86.98 µm. Also, reduction of symptoms of arthritis in FCA- induced arthritic rats was observed after 28 days of topical application of nanoemulsion gel suggesting its potential for topical application [125].

Gokhale et al. formulated topical nanoemulsion gel of quercetin [QCT-NE] for RA management. Optimized nanoemulsion was evaluated for the mean globule size [136.8 ± 1.2 nm], polydispersity index [0.265 ± 0.3] and zeta potential value was -25.4 ± 1.7 mV. At the end of 4 h, 28.23 ± 1.72% of drug was released from free QCT suspension which was considerably lower (p=0.002) than the drug was released from QCT- NE (84.12 ±0.51%) the formulation was also found to be stable for period of study of 90 days. The formulation showed improved stability as well as physiochemical properties of drug. Increased permeation was observed when compared to free gel of quercetin. The paw circumference of CFA control group was 71.21 ± 0.33 mm, which was found to be decreased up to 51.13 ± 1.35 mm with the QCT-NE gel. The inhibition of paw volume by QCT-NE gel was found statistically significant as compared to CFA-control group (p=0.006). This results clearly demonstrated the superiority of Nano emulsion gel over conventional formulation [126].

7.5. Nanomicelles

Nanomicelles are the self-assembling colloidal dispersions of particle size usually within a range of 10 to 100 nm. These systems consist of hydrophobic core and hydrophilic shell and are used as pharmaceutical carriers for hydrophobic drugs to improve their solubility characteristics [127]. Fan et al. prepared novel formulation consisting of curcumin and hyaluronic acid [HA/Cur] spherical nanomicelle for RA having diameter of 164nm. The expression of related cytokines and vascular endothelial growth factor were found to be decreased when administered to RA induced rats. Among four samples, the HA/Cur showed the lowest frictional coefficient of 0.027 ± 0.006 µ, confirming that the nano micelles can significantly decrease the frictional coefficient between the joints. It was also found that the inflammation was reduced from 100 to 60% as seen by paw oedema studies. Also friction between the surfaces of cartilage around the joints was found to be decreased dramatically suggesting the potential of nanomicelle for RA therapy [128].

7.6. Lipid core nanocapsule (LNCs)

LNCs are the nontoxic polymeric nanocapsules consisting of oily core formed by an organogel composed of capric/caprylic triglyceride and sorbitan monostearate. The core is surrounded by the polymeric wall. This chemical composition of core allows these nanocapsules to control the penetration of drug in different tissues. Coradini etal. encapsulated curcumin and resveratrol together in lipid core nanocapsule of particle size around 200 nm with narrow distribution [PDI < 0.1] prepared by interfacial deposition of preformed polymer. The nanocapsules was administered intraperitoneally [i.p.] to arthritic rats. The rats were treated with resveratrol, curcumin, or both in solution or loaded in lipid-core nanocapsules after 14 days of arthritic induction (1.75 mg/kg/twice daily, i.p.), for 8 days. The drugs were not able to decrease pawoedema in solution form but nanoencapsulation improved the antioedematogenic activity of polyphenols at the same doses. In addition, the treatment with co-encapsulated polyphenols showed the most pronounced effects, where an inhibition of 37–55% was observed. The anti-oedematogenic activity was found to improve while no hepatotoxic effects were observed. The authors suggested the LNCs to be a promising strategy for treatment of chronic inflammatory diseases like arthritis [129]

7.7. Solid lipid nanoparticles

SLNs are the new generation colloidal carriers of submicron-sized lipid emulsions in which in place of liquid- lipid, solid-lipid is used. Small size, large surface area, high drug- loading and interaction of phases at the interfaces are the advantageous properties offered by SLNs [130].

Arora etal. prepared curcumin solid- lipid nanoparticles [CUR-SLN] for RA management. The average particle size measured using laser diffraction of C-SLNs was 134.6 ± 15.4 nm. C- SLNs were shown to exhibit the total drug content and entrapment efficiency of 3.78 mg/mL and 81.92 ± 2.91%. Significant ($p < 0.05$) increase in paw volume was observed from day 7 of CFA injection in rats which constantly increased until the 28th day in arthritic rats. Chronic treatment with naproxen (25 mg/kg), free curcumin (30 mg/kg) and curcumin-SLNs (10 and 30 mg/ kg) from day 15 to day 28 significantly ($p < 0.05$) prevented the increase in paw volume in CFA-injected rats, $F(28, 250)=66.9$, $p < 0.0001$ (Fig.3C). Moreover, curcumin-SLNs (30 mg/kg) produced effects comparable to that of naproxen (25 mg/kg). The mean radiographic score on treatment with free curcumin (10 and 30 mg/ kg) reduced to 1.12 and 0.92, respectively, while treatment with curcumin-SLNs (10 and 30 mg/kg) showed enhanced reduction of scores to 0.72 and 0.49, respectively. Confirming the better therapeutic effect of SLNs. further significant reduction in paw withdrawal threshold, joint hyperalgesia, joint stiffness and mobility score was observed. The

authors emphasized the SLN to be a promising and novel approach for improving the biopharmaceutical properties and delivering of drug into the inflamed joints [131].

8. Challenges with novel drug delivery system

Besides the various advantages offered by these novel drug delivery systems, there are some challenges associated with them when it comes to practical applications. The clear definition of these nanoproducts, the characterization with regards to toxicity, safety and lack of effective regulation are some of the limitations. The higher cost and complexity are major challenges. Moreover, the list of approved nanomedicine is large, but their clinical applications are limited due to unavailability of specific regulatory guidelines for the formulations and evaluation of these nanoproducts [132]. As standard protocol is not available, many researchers fail to determine the toxic potential of these nanoproducts in early stages of testing leading to failure in late phases of clinical trials [133,134].

Nanosystems have vast scope therefore there is a need to develop more reformed and integrated regulatory guidelines. For this, cooperation between governments of various key countries is required to develop specific and rigorous protocols that can address the serious safety concerns that will ensure the development of safe and beneficial nanomedicines for the mankind [135,136].

9. Conclusion

There are several anti-arthritic drugs that have been developed and are in market. But prolonged use these drugs may lead to various unwanted side effects. Since in case of RA the treatment is usually required for the entire lifetime therefore natural products can be considered as better approach for its management. Several phytoconstituents derived from natural sources are safe and effective in RA. These phytoconstituents but suffers from various limitations like low bioavailability, less stability and requirement of higher dose. Many literature reports have confirmed the use of nano-system for delivering the phytoconstituents and therefore these limitations can be overcome, and higher drug localization can be achieved for RA treatment.

Nanosystems can improve pharmacological and therapeutic properties of drugs, protect the drug from degradation and are able to deliver the drug to the target site. Furthermore, safety data is required for these nanosytems is required for effective use. In future these nanocarriers can become the first choice for delivery of phytoconstituents for better management of RA.

Declaration of Competing Interest

The authors reflect no conflict of interest.

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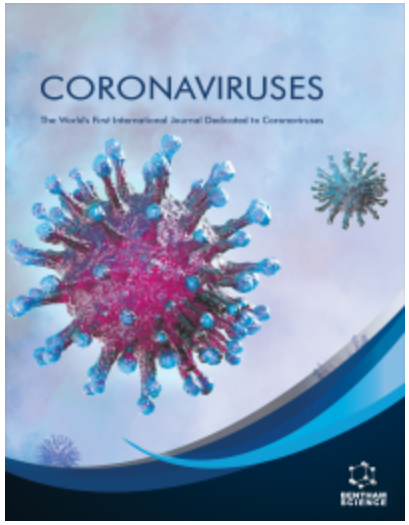

Assessment of Stress, Anxiety, and Depression Levels Among COVID-19 Positive Patients Admitted in Rural Tertiary Care Hospital

Authors: Yadav, Ramakant; Dubey, Neha; Jain, Shweta; Vaidya, Ankur; Kumar, Raj

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Abstract | References | Citations | Supplementary Data

Objective: The aim of the present study was to investigate the psychiatric distress, including stress, anxiety, and depression levels, among COVID-19 positive patients who were admitted between 01 July 2020 to 31 August 2020 to the COVID-19 isolation ward of the Uttar Pradesh University of Medical Sciences, Saifai, Etawah India. Participants included 100 patients, with 55 males and 45 females. The majority of admitted patients (81%) were illiterate. Out of 100 patients, 83 were married, 16 were unmarried, and only 1 was a widow.

Methods: Levels of anxiety, depression, and stress level were noted in admitted patients using Generalized Anxiety Disorder-7 (GAD-7), Patient Health Questionnaire (PHQ-9) depression assessment, and Perceived Stress Scale 4 (PSS-4) assessment techniques, respectively.

Results: Patient Health Questionnaire (PHQ-9) depression assessment results showed minimal, mild, and moderate depression in 9, 25, and 66 patients, respectively, with a 10.6 median score of PHQ-9. Mild, moderate, and severe anxiety (GAD-7 score) was present in 22, 28, and 50 of the patients surveyed. The mean Perceived Stress Scale 4 (PSS-4) was also analyzed, and it reported 6.1 values. The results of the study demonstrated that the patients had a high label of psychiatric distress, but still, admitted patients believe that they will come out from this pandemic condition.

Conclusion: Although patients claimed psychiatric distress and mental health illness, they still denied the requirement of any mental health professionals to minimize stress levels and were satisfied with the medical facilities available in a hospital located in a rural area.

Keywords: Coronavirus (COVID-19); depression; generalized anxiety; perceived stress; psychiatric distress; rural

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Promising Anticancer Activity of β-Carboline Derivatives: Design, Synthesis, and Pharmacological Evaluation

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Abstract

β-carboline consists of a pyridine ring fused to an indole skeleton; it possesses numerous pharmacological activities, including anticancer. Previously, we reported a satisfactory 2D and 3D QSAR study on β-carboline derivatives. Based on QSAR studies, we designed, synthesized, characterized, and screened fourteen β-carboline derivatives for anticancer activity. Eleven of them demonstrated potent anticancer activity against both liver (HepG2) and adenocarcinoma (A549) cell lines. Compound 1-(*N*, *N*-dimethylbenzenamine)-3-(4-(p-tolylmethanimine)-5-thio-1, 2, 4-triazol-3-yl) β-carboline (**9**) was found to be most potent against both cancer cell lines and equipotent towards standard drug Adriamycin. Compounds 1-(p-tolyl)-3-(4-(p-(iminomethyl)-*N*, *N*-dimethylbenzenamine) -5-thio-1, 2, 4-triazol-3-yl) β-carboline (**4**) and 1-(*N*, *N*-dimethylbenzenamine)-3-(4-(m-tolylmethanimine)-5-thio-1, 2, 4-triazol-3-yl) β-carboline (**10**) were found to be 7 to 10 times less potent as compared to Adriamycin against the HepG2 cell line. Molecular docking was also performed with the Glide docking program to explore the binding mode between the synthesized β-carboline derivatives and the receptor CDK2 [1AQ1] protein.

Keywords: β-carboline (/search?q=%CE%B2-carboline); **pharmacophore (/search?q=pharmacophore)**; **QSAR (/search?q=QSAR)**; **[(SW) kNN MFA] (/search?q=%5B%28SW%29+kNN+MFA%5D)**; **docking (/search?q=docking)**; **synthesis (/search?q=synthesis)**; **anticancer activity (/search?q=anticancer+activity)**

1. Introduction

With over 19 million new cases and 9.9 million deaths in 2020, cancer remains a leading cause of premature mortality [**1**]. Numerous novel strategies, including targeted therapies, have been introduced for cancer treatment, but they are also associated with serious limitations, and, therefore, there is still a great need for the discovery and development of new lead small-molecule compounds with increased activity and reduced toxicity towards nonmalignant cells [**2**,**3**].

Natural and synthetic β-carboline alkaloids are well-known planar tricyclic ring structures, possess potential antitumor activity, and can act through multiple mechanisms, including intercalating into DNA [**4**,**5**,**6**] and inhibiting topoisomerase I and II [**7**], cyclin-dependent kinases (CDKs) [**8**,**9**], mitogen-activated protein kinase-2 (MK-2) [**10**], kinesin-like protein Eg5 [**11**], and I-kappa-B kinase (IKK) [**3**]. DNA intercalation and topoisomerase I inhibition were thought to be the primary mechanisms of

carbolines' antitumor activity [**12**,**13**]. To date, numerous researchers have reported a number of β-carboline derivatives that possess anticancer activity [**14**,**15**,**16**,**17**].

Rational drug design identifies new bioactive compounds with favorable properties from the total chemical space. This often implies knowledge of the target, usually a protein, to find new ligands. These ligands do not necessarily originate from a design process but can also branch from a virtual screening of compound libraries [**18**]. The present research group performs and reports a number of QSARs and drug design studies, and reports the numbers of compounds that possess considerable biological activities, including anticancer activities [**19**,**20**,**21**,**22**]. We previously conducted 2D and 3D QSAR studies on β-carboline derivatives using the V-Life Science molecular design software and PHASE (Schrödinger) [**13**]. The study revealed highly predictive 2D QSAR and atom-based 3D QSAR models. The 2D QSAR studies signify the positive contribution of the hydrogen count (-NH_2, -SH, groups) and SaaaCE index (thiadiazole, oxadiazole groups) towards the biological activity. Moreover, 3D QSAR studies suggested the favorability of bulky groups (naphthyl, 4-dimethylaminobenzyl, benzotriazole groups) in the R_1, R_2 positions for producing potent compounds for better activity (**Figure 1**).

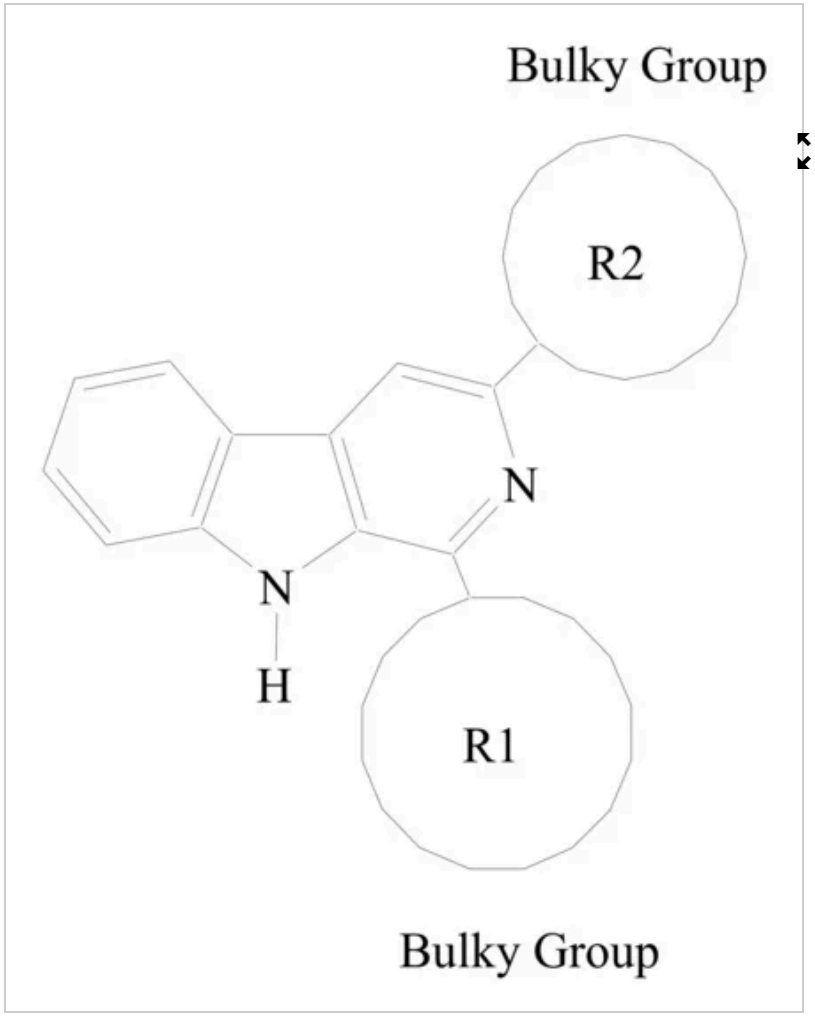


Figure 1. Structure of β-carboline with bulky groups at R1 and R2 positions for anticancer activity.

Studies were further extended by our research group and 2D and 3D QSAR studies on different data sets of β-carboline derivatives were reported [**23**]. The results revealed that the 2D QSAR studies signify a positive contribution of the carbon count (benzyl, naphthyl, octyl groups) and SsCH3 count (methyl, acetyl groups) towards the biological activity, whereas there is a negative contribution of the oxygen count (hydroxy groups) towards anticancer activity. Moreover, 3D QSAR studies suggested the favorability of bulky groups (3-benzyl-4H-pyrazole, naphthyl groups) at R_2 positions for producing potent compounds for better activity (**Figure 1**). These new 2D QSAR and atom-based 3D QSAR models provide us with valuable

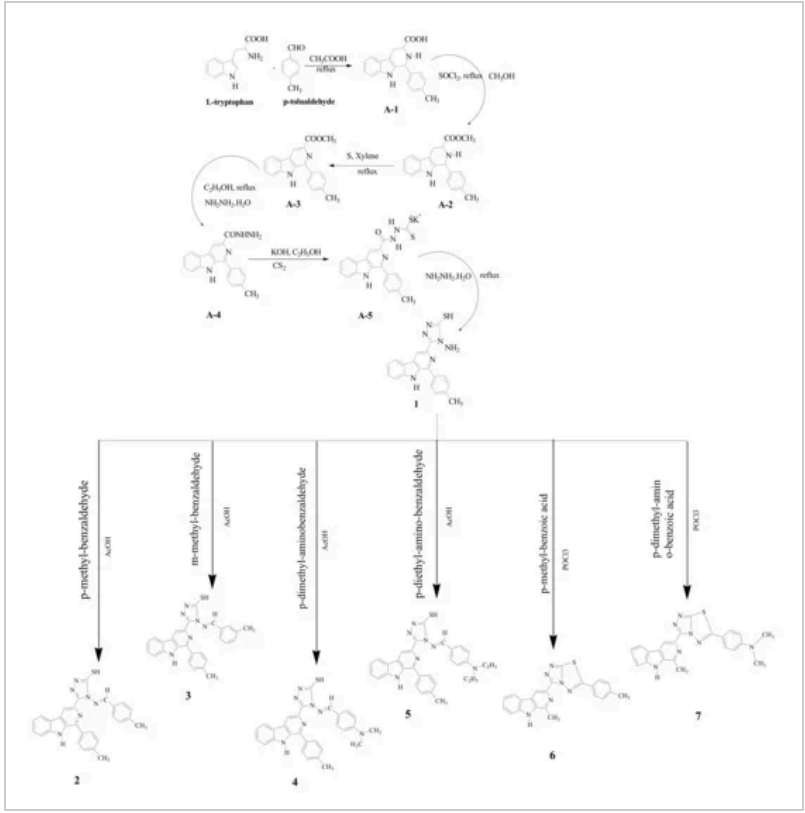
information and insights into the structural requirements of novel β-carboline derivatives as antitumor agents. Based on these QSAR results, we have reported the synthesis and anticancer activity of some novel β-carboline derivatives. Furthermore, docking studies are also reported, which explore the binding mode between the synthesized compounds and the protein molecules.

2. Materials and Methods

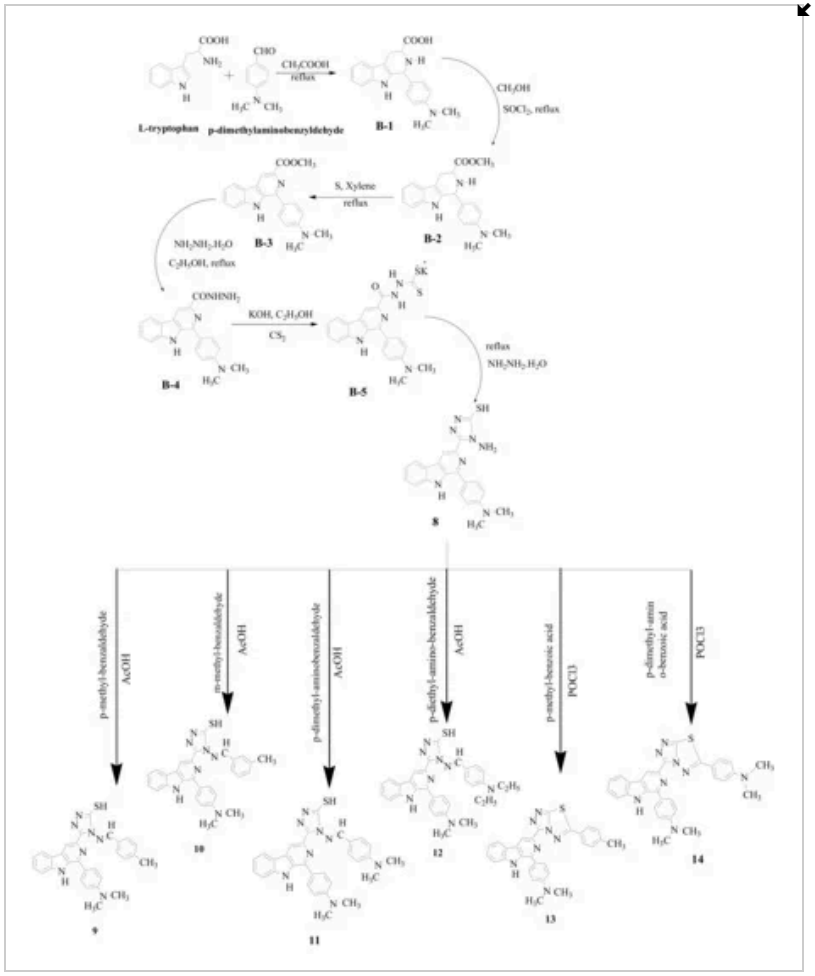
2.1. Synthesis of β-Carboline Derivatives

In this work, a number of new β-carboline derivatives were designed and synthesized. All the chemicals for synthesis were purchased in the highest available quality from commercial suppliers (Sigma-Aldrich, Merck Ltd., Mumbai, India) and used without further purification.

The synthetic routes for the preparation of β-carboline derivatives are presented in **Scheme 1** and **Scheme 2**.



Scheme 1. Synthetic scheme for β-carboline derivatives via the reaction of starting material L-tryptophan and p-tolualdehyde.



Scheme 2. Synthetic scheme for β-carboline derivatives via the reaction of starting material L-tryptophan and p-dimethylaminobenzyldehyde.

In **Scheme 1**, the methyl tetrahydro- β-carboline-3-carboxylates (**A-2**) were prepared through the Pictet–Spengler condensation of L-tryptophan with p-tolualdehyde in acid medium and subsequent esterification of the carboxylic acids with methanol and thionyl chloride. The conversion of the derivatives to the corresponding β-carboline-3-carbohydrazides (**A-3**) was

carried out by oxidation with sulfur in refluxing xylene of methyl-1,2,3,4-tetrahydro-9H-β-carboline-3-carboxilates, followed by the reaction of methyl-β-carboline-3-carboxylates with hydrazine hydrate, in ethanol under reflux (yield **A-4**). The acid hydrazides (**A-4**) were allowed to react with carbon disulfide in the presence of potassium hydroxide in ethanol to afford the corresponding intermediate potassium dithiocarbazinate (**A-5**). This salt underwent ring closure with an excess of 99% hydrazine hydrate to give a 4-amino-3-substituted-5-mercapto-(4H)-1,2,4-triazole β-carboline derivative (**1**). The resulting triazole-carboline derivatives were then converted to 1,2,4-triazolo-[3,4-b]-1,3,4-thiadiazoles (**2**–**7**) in a one-pot reaction with aromatic acids and phosphorus oxychloride.

Similar to **Scheme 1**, in **Scheme 2**, the reaction is carried out through the Pictet–Spengler condensation of L-tryptophan with another aldehyde (i.e., p-dimethylaminobenzyldehyde). Furthermore, all the compounds reported in **Scheme 2** were synthesized in a similar fashion as in **Scheme 1**. The detailed synthesis procedures of compounds 1–14 are given in the **Supplementary Materials**.

All the synthesized compounds were tested for their purity by TLC using the solvent system $CHCl_3$:CH_3OH (15:1) as a mobile phase and silica gel G precoated aluminum sheets (60 F254, Merck) as a stationary phase. The melting points of the synthesized compounds were determined by the open capillary method using the Toshniwal melting point apparatus. The proton NMR and ^{13}C NMR spectra of the synthesized compounds were recorded on the Bruker Avance II 400 NMR spectrometer as solutions in $CDCl_3$ or DMSO-*d6* using TMS as an internal reference, and chemical shift values are expressed in *δ* units. The IR spectra of the synthesized compounds were recorded on an IR spectrophotometer (Jasco, FT/IR-4100 type A) in KBr phase. The mass spectra of the synthesized compounds were recorded on an FAB mass spectrometer (Jeol SX102-FAB).

2.2. Biological Screening

In the present study, all the synthesized compounds were subjected to growth inhibition assays in different cancer cell lines (A-549 and HepG2). At 540 nm, the optical density (OD) was measured using an ELISA reader. The optical density (OD) of *sulforhodamine B* (SRB) in each well was directly proportional to the cell number, so the OD values could be plotted against the concentration and the IC_{50} determined by using a program such as Graph-Pad PRISM [**24**].

2.3. Docking Studies of Designed Compounds

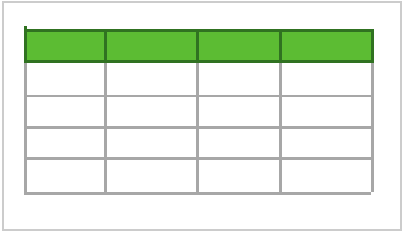
Using the docking program Glide, the researchers investigated the appropriate binding orientations and conformations of the synthesized β-carboline derivatives interacting with cyclin-dependent kinases (CDKs). Glide is a fast, flexible docking

method that uses an incremental construction algorithm to place ligands into active sites. By default, the docking program produces 10 docked structures for each β-carboline derivative. The conformation with the lowest docking energy in the most populated cluster is selected as the possible "active" conformation against the CDK2 [PDB: 1AQ1] active site [**25**,**26**,**27**]. In the present study, 14 compounds were successfully docked into the 1AQ1 site. The detailed procedure of docking studies is reported in the **Supplementary Materials**.

3. Results and Discussion

The novel β-carboline derivatives designed are shown in **Table 1**. These designed compounds were synthesized and characterized via spectroscopic techniques, and evaluated for their anticancer activity. The spectroscopic data revealed the successful synthesis of the designed compounds, and the anticancer activity was shown to be significant as compared to that of marketed ones.

Table 1. Newly designed, substituted β-carboline derivatives.

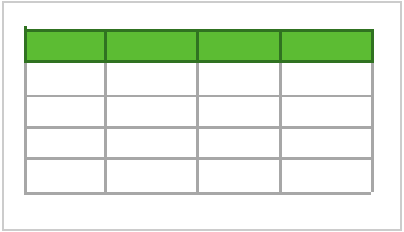


The synthesis procedures are depicted in **Scheme 1** and **Scheme 2**. Compounds **1**–**14** were synthesized by the reaction of starting material L-tryptophan with p-tolualdehyde or p-dimethylaminobenzyldehyde in the presence of acetic acid. The products were obtained in a 65–80% yield. These were found to be stable toward air and moisture at room temperature. All the synthesized derivatives showed moderate to high solubility in various organic solvents, such as methanol, chloroform, acetone, and dimethyl sulfoxide, but were insoluble in water. The spectral data matched the predicted structures of the synthesized compounds. In the ^{1}H NMR, all protons were in their predictable regions, with integral area ratios per group conforming to the predicted number of protons per group. In the ^{13}C NMR, the peaks of each group were consistent with the theoretical prediction of the number of carbon atoms in the structure. Elemental analysis confirmed the elemental composition of C, H, and N in the synthesized compounds.

3.1. Biological Screening Results

Except for (**6**), (**7**), and (**13**), all of the synthesized β-carboline derivatives inhibited various cancer cell lines effectively (**Table 2**). Compound (**9**) showed the utmost activity against both liver (HepG2) and adenocarcinoma (A549) cancer cell lines and was found to be roughly as equipotent as Adriamycin. Compounds (**4**) and (**10**) were found to be approximately 7–10 times less potent as compared to Adriamycin against the HepG2 cell line. The majority of compounds were active but approximately 50–100 times less potent than Adriamycin against both the HepG2 and A549 cancer cell lines.

Table 2. In vitro cytotoxic activity of synthesized compounds by SRB assay with their dock score or G-score.



For most compounds, drug sensitivity for both cell lines (HepG2 and A549 cells) was nearly equal, and the anticancer activity (IC_{50} value) was almost equally dependent on the type of aromatic ring on the ligand. The presence of the 4-methanamine group promotes biological activity, whereas β-carboline substituted with 1,4 triazolo (3,4-b)-1,3,4-thiadiazole is either inactive or less potent. It has been reported that 1-(N,N-dimethylbenzenamine)-substituted β-carboline derivatives are more potent than 1-(p-tolyl)-substituted β-carbolines. Compounds (i.e., **6**, **7**, and **13**) containing a 1,3,4-thiadiazole-fused ring are biologically inactive (except compound **14**). The imino moiety is conducive to biological activity and the three most active compounds (i.e., **4**, **9**, and **10**) are imino derivatives.

3.2. Docking Results

The in silico (docking) studies distinguished the compounds' hypothetical binding modes using the X-ray crystal structure of CDK2 [PDB ID: 1AQ1] and G-score, as shown in **Table 2**. The top docked conformations (poses) closely resembled the co-crystallized conformation, with a root-mean-square deviation (RMSD) of 1.07–1.70 in the non-hydrogen atomic positions of the ligand.

Hydrogen bonding is an important factor that causes bonding with hetero atoms, so the docking interactions of the most active compound (**9**) with 1AQ1 were shown through hydrogen bonding, as seen in **Figure 2**. The hydrogen bond was found between the residue of compound (**9**) and the 1AQ1 in the R_2 position. Furthermore, the sulfur atom of the triazole ring of compound (**9**) exhibited a van der Waals interaction with the amino acid residues, such as His84, at a distance of 2.68895 Å. Docking interactions were also discovered between compounds (**1**–**14**)'s residues and the 1AQ1. The docking results showed that the binding mode of β-carbolines of compound (**9**) with CDK2, dock score, and hydrophobic cavity included His-84, Gln-131, and Asp-86 amino acid residues; results are shown in **Figure 2** and **Figure 3**. These interaction results revealed the possible binding of a target molecule to CDK2 and the further development of novel compounds for antitumor activity.

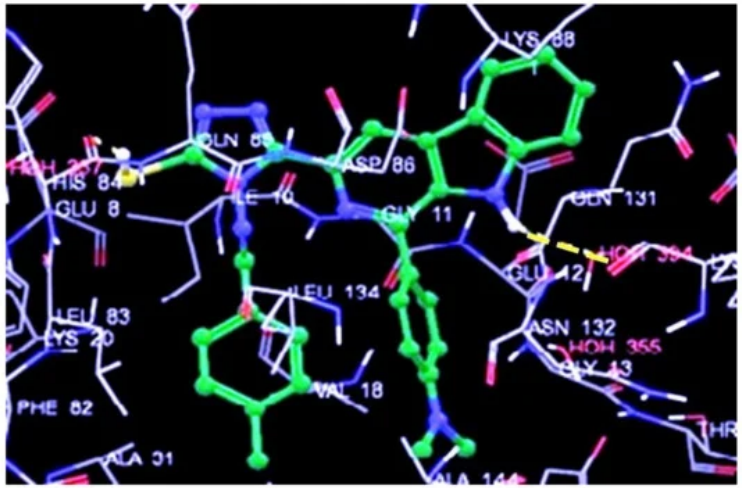


Figure 2. Docking conformation in the active site of CDK2 of most active compound (**9**) in the context of hydrogen bonding is displayed as dotted yellow lines.

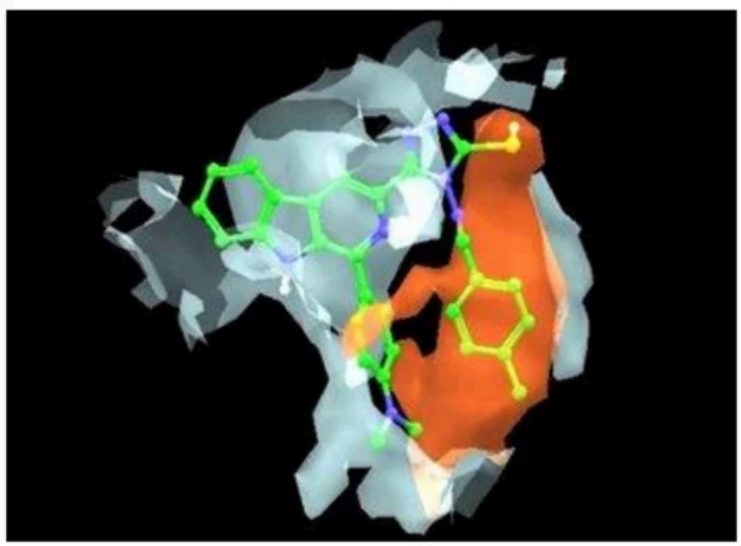


Figure 3. Docking conformation in the active site of CDK2 of most active compound (**9**) in the context of hydrophobic region.

Compound (**9**) showed the highest dock score or G-score and this suggested that the docking interactions of compound (**9**) in 1AQ1 binding sites may be responsible for its highest biological interaction, followed by compounds (**4**) and (**10**). A linear correlation between G-score and biological activity was observed.

The correlation between the biological activities (pGI_{50} for HepG2 cell line) of the synthesized compounds and their dock scores in Glide docking is shown in **Figure 4**, which shows a linear correlation. The docking study gives only a rough approximation of the kinase inhibition activities of the synthesized compounds, and enzyme-based kinase (CDK2) inhibition experiments are required in the future.

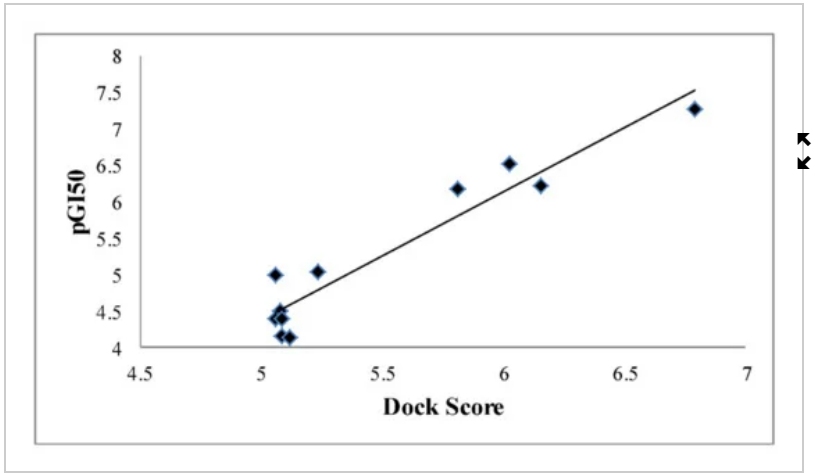


Figure 4. Correlation between the biological activity of synthesized compounds and their dock scores in Glide docking.

4. Conclusions

With the aim of developing potent anticancer compounds, we have previously performed and reported 2D and 3D QSAR models on β-carboline derivatives, which provided useful information and insights into the structural requirement for anticancer activity. On the basis of QSAR outcomes, new, potent compounds were designed, synthesized, and characterized using FT-IR, ^{1}HNMR, ^{13}CNMR, FAB-MS, and elemental analysis techniques. These synthesized compounds were assayed for their in vitro biological activities, which showed that compound (**9**) was the most potent against the HepG2 and A549 cancer cell lines as compared to Adriamycin. All the synthesized compounds docked well into the binding pocket of the target protein CDK2 [1AQ1] and interacted with the crucial amino residues. The docking studies revealed a linear correlation between the docking score and anticancer activity, which suggested that the binding interaction of compounds with the active site of target protein 1AQ1 may be responsible for their anticancer activity.

Supplementary Materials

The following supporting information can be downloaded at **https://www.mdpi.com/article/10.3390/chemistry4040091/s1 (https://www.mdpi.com/article/10.3390/chemistry4040091/s1)**. Supplementary File contains detailed procedures for

synthesis of compounds and spectroscopic spectra of final compounds, and also contains detailed procedure for docking studies.

Author Contributions

Conceptualization, A.V.; methodology, R.K.A.; software, R.K.A.; validation, R.K.C.; formal analysis, R.K.C.; investigation, R.K.C.; resources, R.K.A.; data curation, R.K.C.; writing—original draft preparation, R.K.C.; writing—review and editing, A.V.; visualization, R.K.C.; supervision, A.V.; project administration, A.V.; funding acquisition, A.V. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data available in article and raw data are available from the corresponding authors upon request.

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Conflicts of Interest

There is no conflict of interest.

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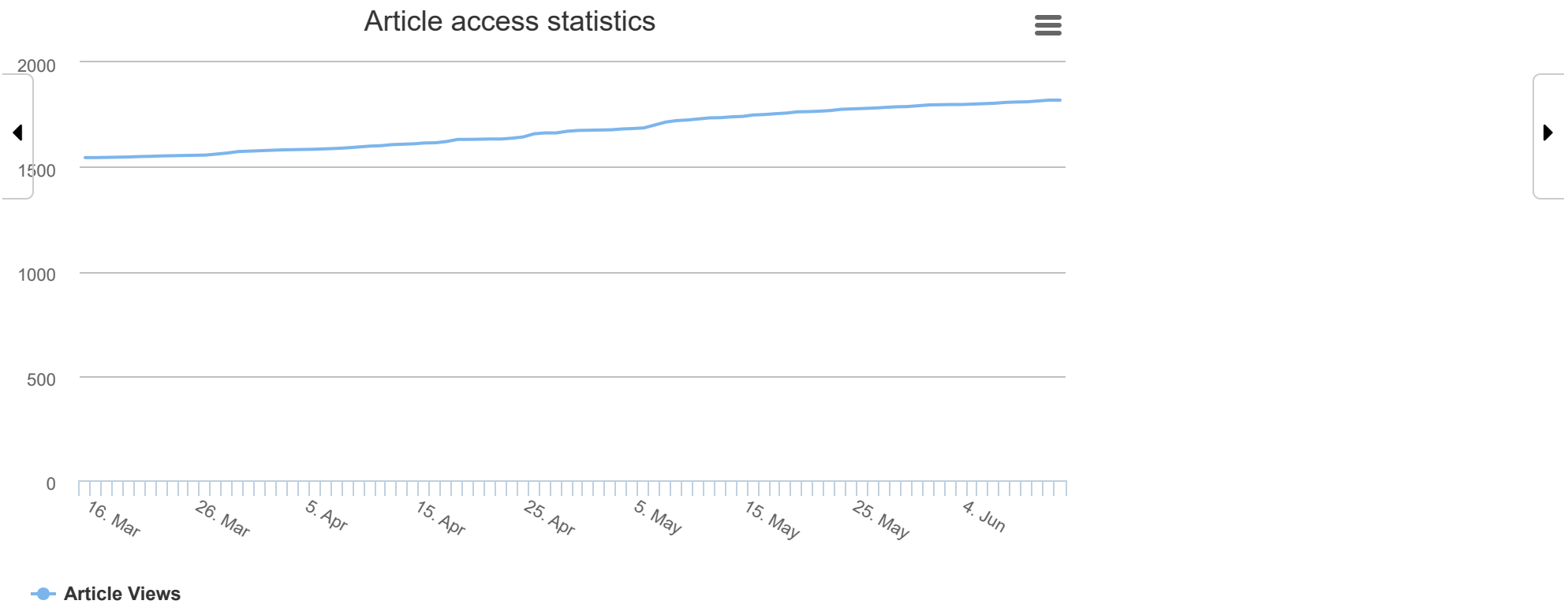

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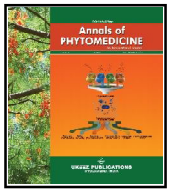

Review Article : Open Access

Recent advances in isatin-thiazole hybrids as potential anticancer agents

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Abstract

Cancer has become a rapidly expanding global threat. In current review, we examine the finding of derivatives based on isatin, thiazole and isatin-thiazole hybrids that have already been identified as anticancer agents. Isatin and thiazole derivatives can be found from natural resources, whereas isatin is found in human fluids for the metabolism of amino acid. Various isatin derivatives such as thiosemicarbazones, hydrazones, imines, among other heterocyclic moieties were screened for different anticancer effects. Few isatin derivatives have trail in pre-clinical and clinical screening as angiogenic inhibitors. Isatin hybrids and thiazole derivatives presented promising antineoplastic properties against different cancer cells by acting on various macromolecules. They also disclose several methods of action such as producing reactive molecules, for oxidative damage, target DNA and restrict few properties. The review emphasizes advances in the development of isatin, thiazole and isatin-thiazole hybrids as anticancer agents.

1. Introduction

Cancer has become a rapidly expanding global threat. According to the global health observatory report of WHO 2020 states that, over 10 million people lost their life due cancer worldwide. It is expected that till 2030, 26 million fresh cancer cases diagnoses and 17 million people loss their life due to cancer worldwide, with an estimated 2.3 million fresh cases of cancer. Female breast cancer the most commonly diagnosed cancer as it surpassed lung cancer (Ferlay *et al.*, 2020). Although, cancer chemotherapy has made significant advances in current years, but there is still a significant unmet demand of novel anticancer drugs having high potential effect, target selectivity, and low toxicity (Stewart and Wild, 2015). Therefore, there is required to explore new anticancer agents. Cancer can begin nearly anywhere in the human body, the cancer results from the DNA mutation, the instructive cells grow out of control (Taha *et al.*, 2019). It is a hereditary disorder caused by changes in genes, specifically tumour suppressor genes, DNA repair genes, and protooncogenes, which affect how cells behave, primarily how they grow and divide (Uddin and Veeresh,2020). Genetic changes can be happened because of error during cell division, detrimental substances in environment such as UV rays, chemicals in tobacco or they may be inherited from our parents (Shegokar and Sawant, 2014).

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Isatin (1H-indole-2,3-dione) has been recognized for around 150 years. It is an important nitrogen-containing aromatic heterocyclic chemical found in numerous plants as well as an endogenous polyfunctional heterocyclic compound with biological action in mammals (Medvedev *et al.*, 2019; Gezici, 2018). Over the last decade, isatin has gained attention as a helpful nucleus in medicinal chemistry and drug development. Previous research indicates that indole-2,3 dione andindole-2,3 dione containing derivatives show a broad range of pharmacological actions, *viz.*, antineoplastic (Wang *et al.*, 2017), antidiabetic (Xie *et al.*, 2017), antimicrobial (Srivastava *et al.*, 2020), anticonvulsant (Nikalje *et al.*, 2015), antibacterial (Chemchem*et al.*, 2020), anti-inflammatory (Lahari *et al.*, 2020), antiviral (Kumar *et al.*, 2021). However, only a few isatin derivatives have received clinical approval, including semaxanib, nintedanib, sunitinib, orantinib and toceranib (El-Naggar *et al.*, 2018).

Thiazole and its derivatives, on the other hand, are regarded as a key sulphur and nitrogen heterocyclic chemical with a broad range of pharmacological actions such as anticancer (Bera *et al.*, 2022; Sekar *et al.*, 2010), antimicrobial (Althagafi *et al.*, 2019), antiviral (Abdel-Latif*et al.*, 2021) and anti-epileptic (Mishchenko *et al.*, 2020)tiazofurin (Franchetti*et al.*, 1995), dasatinib (Li *et al.*, 2009), dabrafenib and ixabepilone (Yao *et al.*, 2014) are also thiazole-containing medicines that have been identified to be involved in the treatment of cancer, many of the derivatives are commercially available as anticancer therapies.

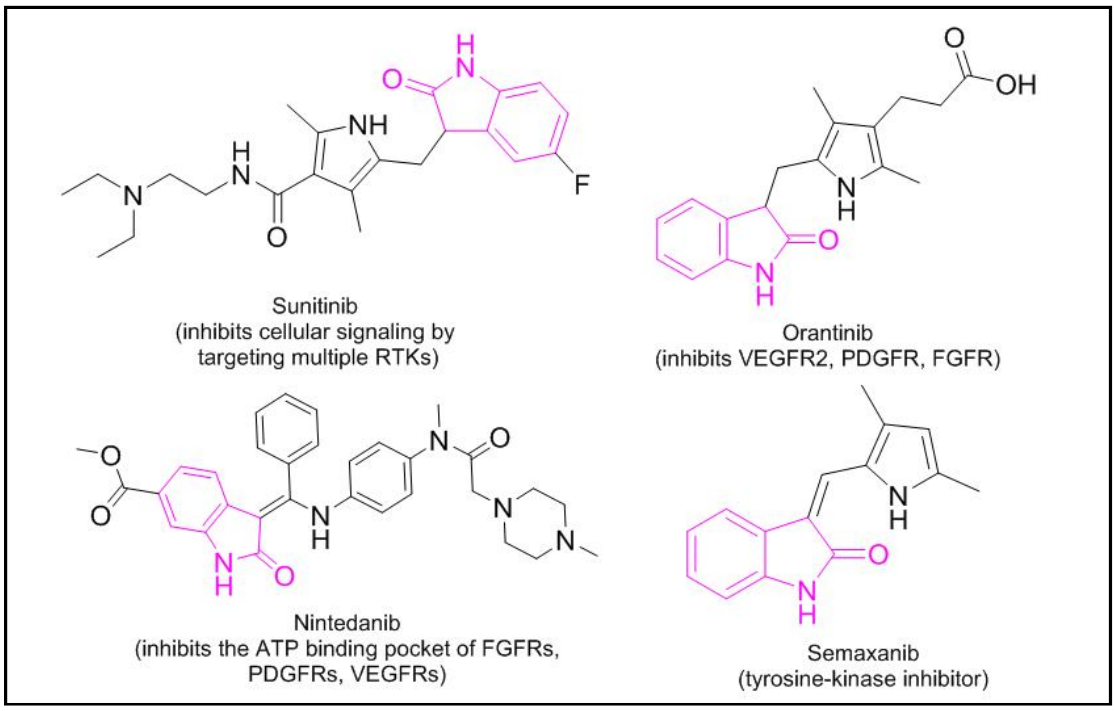


Figure 1: Clinically approved isatin-bearing anticancer drugs.

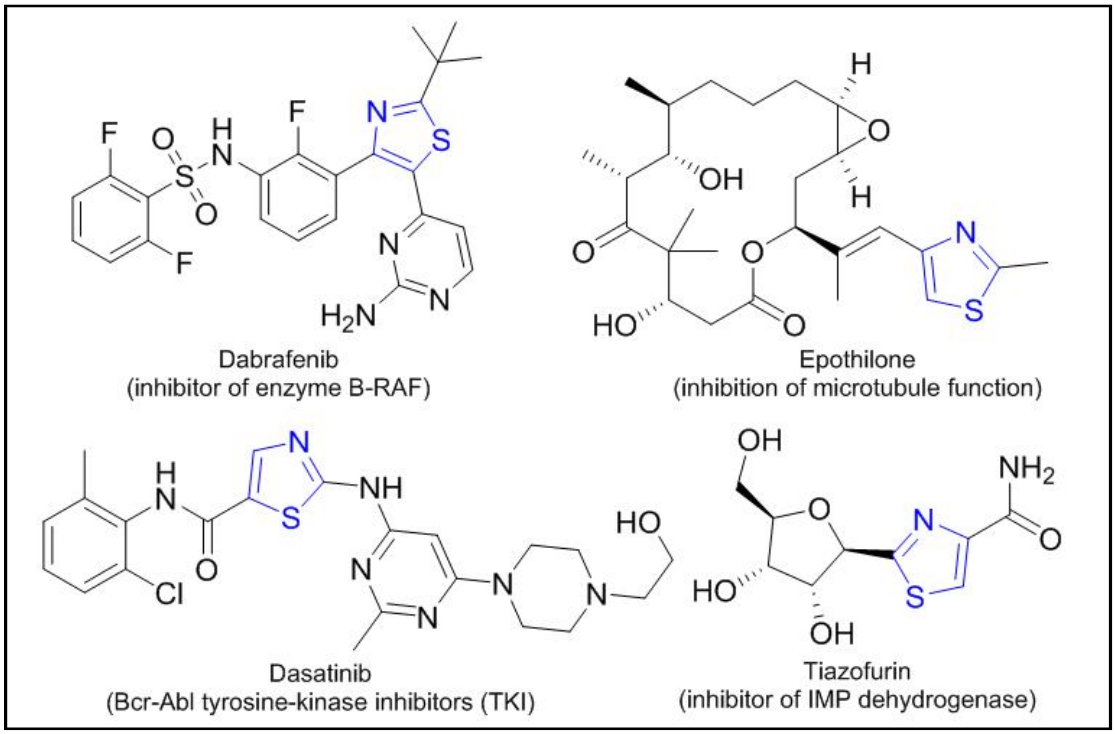


Figure 2: Clinically approved thiazole-bearing anticancer drugs.

Researchers have discovered some promising chemical architectures containing two or more biologically active pharmacophores using molecular hybrid-based approaches over the years (Viegas *et al.*, 2007; Mishra *et al.*, 2016). Furthermore, these hybrid molecules typically have more than one mechanism of action which may cause decreased adverse effect, enhance pharmacodynamic with pharmacokinetic features, improve efficacy, overcome drug resistance. *etc.* So, the review emphasizes advances in the development of isatin-thiazole hybrids as anticancer agents.

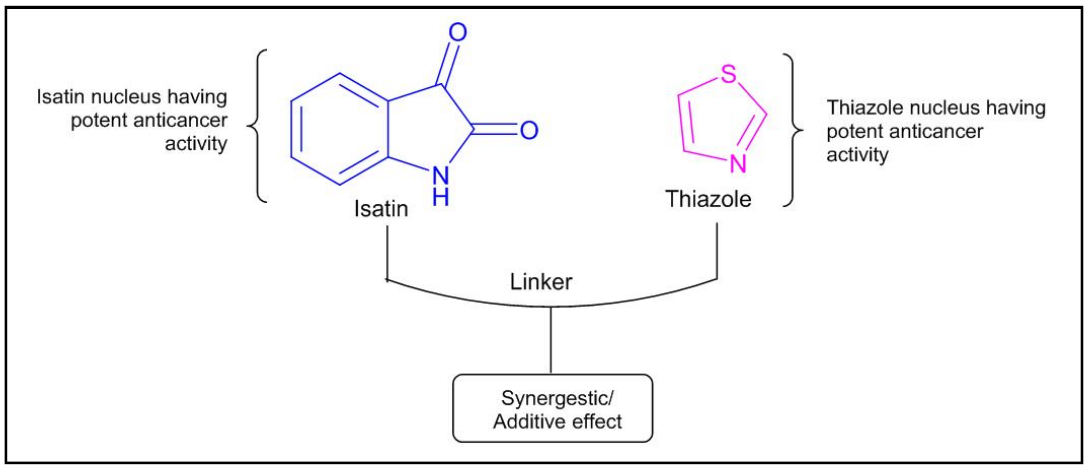


Figure 3: Isatin-thiazole containing hybrids.

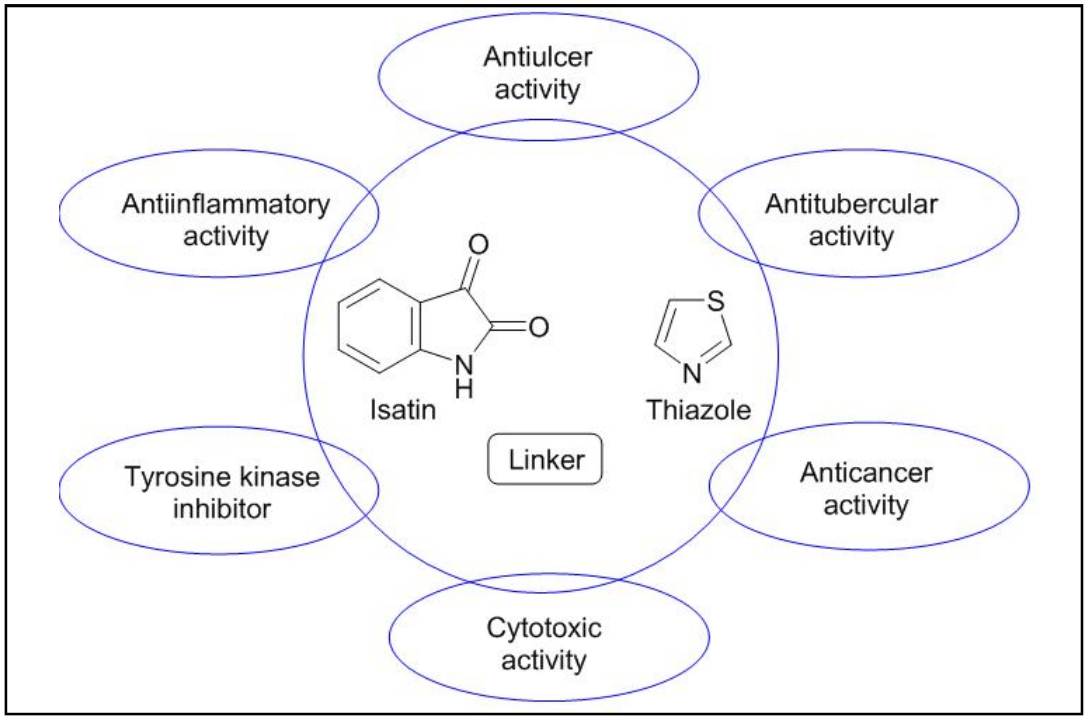
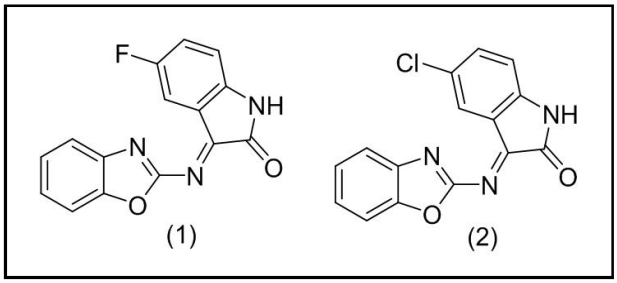


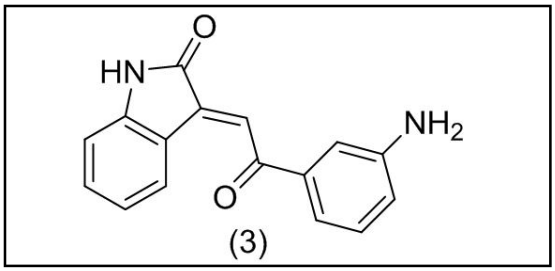
Figure 4: Biological characterization of isatin-thiazole hybrids.

2. Isatin as anticancer agent

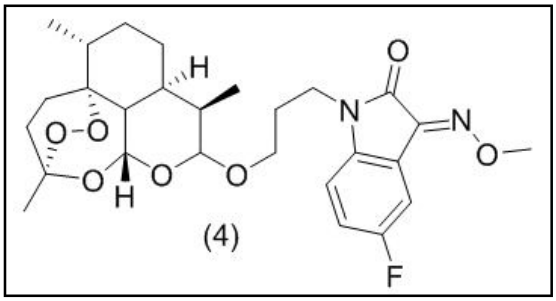
A series of benzoxazole-isatin derivativeswas synthesizedand evaluated by Susithra *et al. (*2022) using the MTT method on HeLa, IMR-32 and MCF-7 cancer cell lines using cisplatin as standard and also docked with Telomerase (5CQG) and GlcN-6-P synthase (2VF5). The result shows that compounds1 and 2 substituted with chlorine and fluorine group at 5thcarbonshows ahigh potential with a dock score value of -7.56 and -7.97.



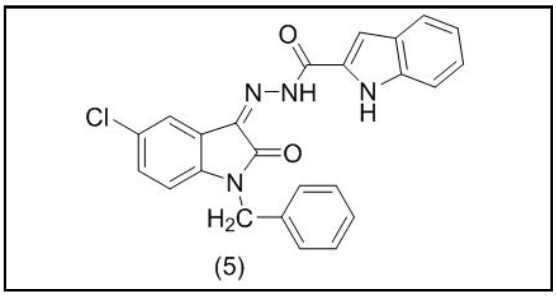
A set of isatin hybrid with a, b-unsaturated ketone was designed and developed using the association principle by Wang *et al.* (2017). These isatin hybrids were tested for their cytotoxic action on diverse cell lines, *viz.*, SGC-7901, BGC-823 and NCI-H460 by MTT assay. Compound **3** inhibited proliferation in all of the cancer cells examined and was found to be highly effective on the NCI-H460 cell lines, with an IC_{50} of 3.2 μM.



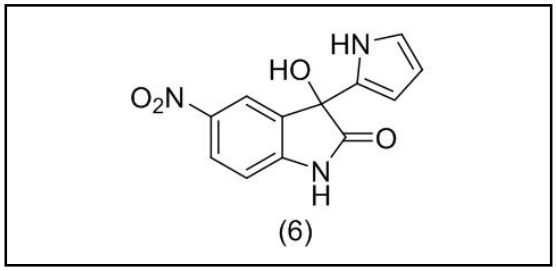
In another report nineteen propylene-tethered dihydro artemisinin-1*H*-indolin-2,3-dione, hybrids of 1*H*-indolin-2,3-dione was synthesized, by Zhang *et al.* (2022). Allsynthesized derivatives were tested *in vitro* antiproliferative screening against three different lung tumor cell lines. Among them, hybrid compound**4** expressed an excellent effect on resistant lung adenocarcinoma cell line, lung carcinoma epithelial cells line and lung adenocarcinoma cell line having an IC_{50} of 21.7-28.9 lμM.



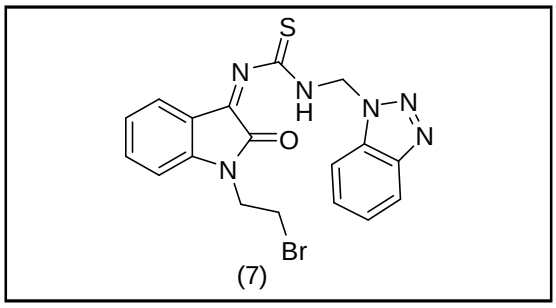
A series of different substituted isatin-indole conjugates was developed and tested for their *in vitro* anticancer activityon three cancer cell lines by (Al-Wabli*et al.*,2021). Esterification of indole-2-carboxylic and subsequently hydrazinolysis using hydrazine hydrate gives the intermediated which was treatedwith various substituted isatin derivatives to give novel isatin-indole derivatives. The antiproliferative activity of these derivatives was evaluated with A-549, ZR-75 and HT-29 tumor cells lines, in which compound **5** showed potent *in vitro* antineoplastic effects against all three cancer cell lines having an IC_{50} of 1.17.



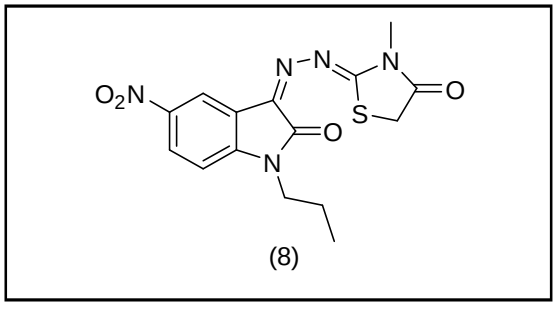
Santoso *et al.* (2021) evaluated *in vitro* cytotoxicity against HepG2 cell lineof a series of isatin-pyrrole scaffolds. The MTT method was used to perform cytotoxicity activity with HepG2 cells, the study reveals that isatins conjugated with pyrrole may give some potent anticancer drugs as the compound **6** of the synthesized series bearing nitro group showed the maximum cytotoxicity activity with an IC_{50} of 0.47 mM.



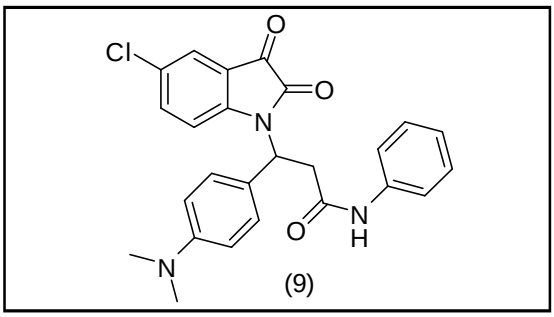
In another report (Kumar*et al.*, 2020) synthesized imidazole-linked isatins *via* condensing imidazole with various substituted isatins. The MTT assay on breast tumor cell line (MCF-7) and MCF-10A as a control, of synthesized scaffolds **7** showed a relatively potent activity caused by about 40% cell antiproliferation at 0.75 ¼M in MCF-7 cells,with 70% cell survival reported at 8.0¼ M in MCF 10 A cells, also the docking result of **7** shows the highest binding affinity towards phosphoinositide 3-kinases.



Yousef *et al.* (2020) synthesized thiazolidinenes and thiazolidinone-linked isatins and evaluated cytotoxicity against various cells, *viz.*, HepG2, MCF-7, HT-29. The MTT assay shows that the E-conformer of derivative **8** bearing nitro group was found to be more potent than doxorubicin against HepG2. Although, authors revel that thiazolidinenes-isatin scaffolds were found to be more potent than thiazolidinone. Also, the docking result shows that nitro-substituted isatin derivatives have a high binding affinity towards cyclin-dependent kinase 1.

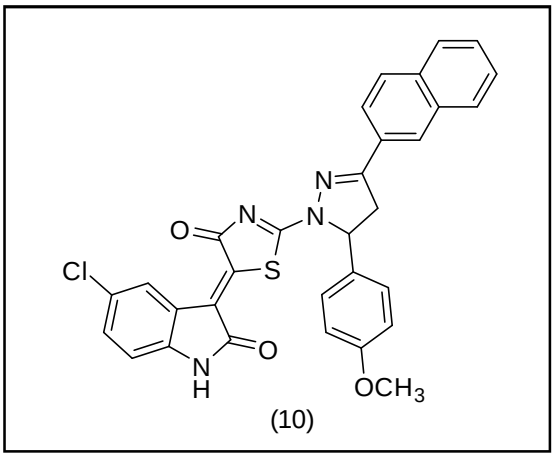


Deepthi *et al.* (2022) synthesized and tested a novel set of mannich bases of isatin derivatives as potent antiproliferative agents using MCF7 cell lines. The MTT assay shows that derivative **9** has more activity against MCF-7 cells incomparison with doxorubicin as a standard drug with an IC_{50} value of 41.65 ¼ M.



(9)

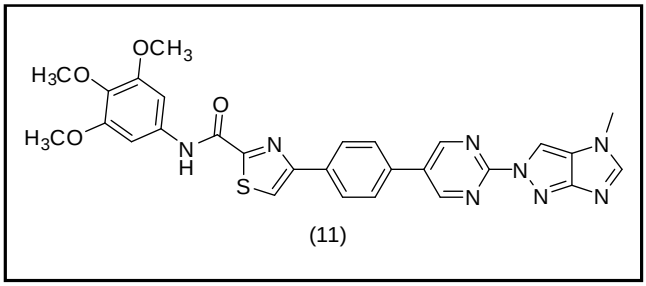
In another report (Meleddu *et al.*,2019) reported their investigation onisatin-dihydropyrazole hybridsfor their anticancer activity against different cell lines (IGR39, A549, MDA-MB-231, U87, BT474, MCF-7, SKOV-3, BxPC-3 and H1299). The study shows that derivative **10** containing methyl group in the 5thposition of the is atin nucleus is the most promising among all synthesized derivatives against IGR39, U87 and IGR39 compared to standard drug sunitinibhaving EC_{50} values range of 0.01 to 0.38 mM.



(10)

3. Thiazole as anticancer agent

Bandaru *et al.* (2021) developed and synthesized a series of new amine-based thiazole-pyrimidine derivatives *via* linking with fused imidazole-pyrazole derivatives and screened them against different human cancer cells lines, A2780 (ovarian), Colo-205 (colon), A549 (lung), MCF-7 *via* technique. Compounds with di/tri-substituted were found to be more potent as compared to mono-substituted compounds. Two derivatives **11** and **12** were dispatched with maximum anticancer effect compared to standard drug etoposide tested on all the cancer cell lines, also possible protein binding was identified. The comparative studies of violine plot and binding energies suggested that analogues were having potent binding sites at ATR kinase.



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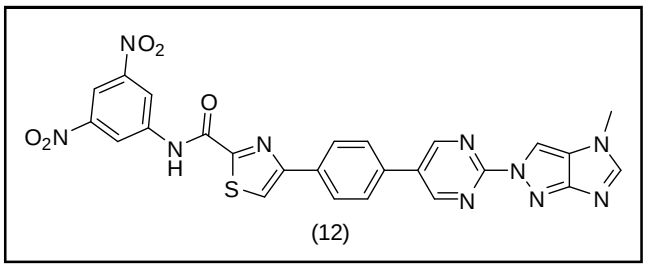
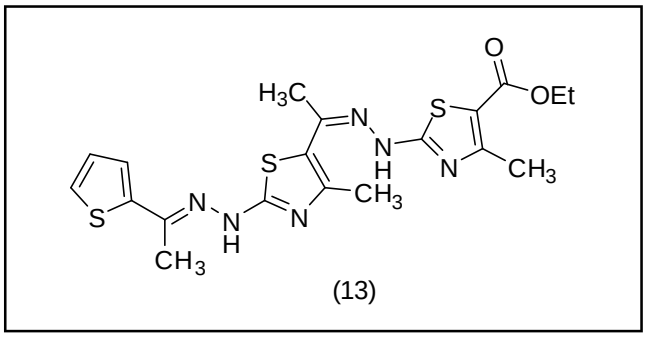


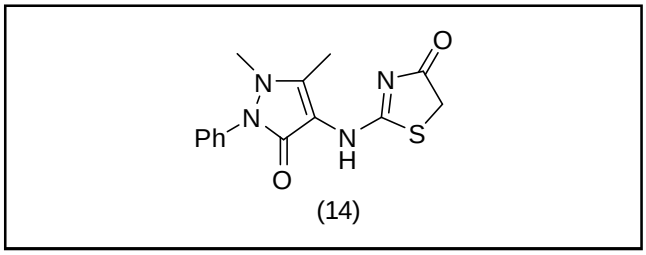
(12)

Gomha *et al.* (2021) developed a fresh series of 5-(1-(2-(thiazole-2-yl) hydrazone) ethyl) thiazole analogs. All derivatives were evaluated for anti-neoplastic activity using an MCF-7 cell line *via* MTT assay. It was found that analog **13** produces maximum activity having IC_{50}value of 14.6 mM as compared to cisplatin. Molecular docking studies were performed on the Rab7b target protein site and withthe help of the admet SAR tool *in silico* studies like toxicity and ADME were carried out.



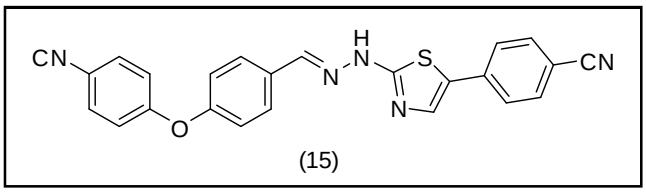
(13)

Othman *et al.* (2022) synthesized two series of pyrazoline-3-one linked with thienol (3,2-d) thiazoleordihydrothiazolo (4,5-d) thiazole scaffold *via* an N-H linker by using pyrazolinone-thiazolinone analogues as important antecedent. All the synthesize danalogues were screened for anti-proliferative effects on two different cancer cell lines, *viz.*, HepG-2 and MCF-7, analog**14** expressed the most anticancer effect on both the cell lines, further these compounds were also tested as multi-targeting kinase inhibitors on normal human cell line WI-38.



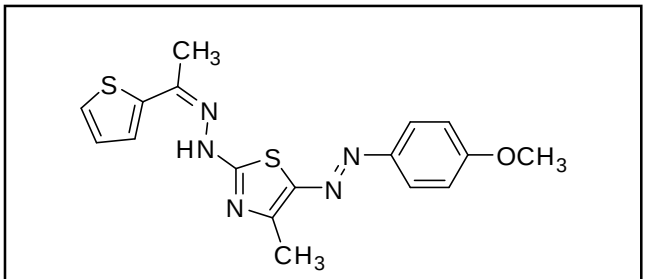
(14)

In another study (Altýntop *et al.*, 2018) reported a series of new thiazole derivatives and all the synthesizedan alogues were evaluated for anticancer effect at C6 rat glioma, A549 and NIH/3T3 (healthy) mouse embryonic. Among all the compounds, derivative **15** shows 45 and 71% inhibition on A549 and C6 cell line, respectively. In addition, the molecular docking study on serine/threonine kinase 1 (AKT1) enzyme shows satisfactory binding.



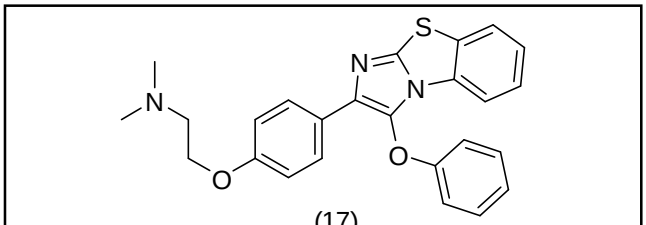
(15)

El-Naggar *et al.* (2022) synthesized various derivatives of hydrazinyl thiazole derivatives. All freshly synthesized derivatives were screened for *in vitro* antineo plasticaction against colorectal cancer HCT-116, HePG-2 and MCF-7 cell lines using the MTT technique. The synthesized scaffold **16** showed maximum activity with IC_{50} values of 3.81, 7.19, 8.22 ㎜ for HePG2, MCF-7 and HCT-116 respectively compared to roscovitine as standard drug although, chloro and methoxy substituted compounds also showed selective against HCT-116 and HePG-2 cell line.



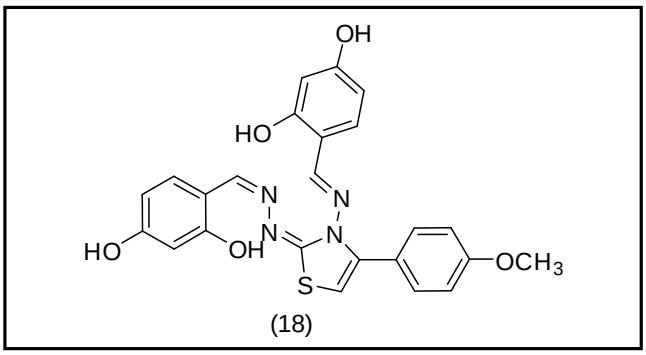
(16)

In another report (Ahmadi *et al.,* 2022) designed and synthesized novel derivatives of diaryl benzo [d] imidazo [2,1-b] thiazole analogues having aminoethoxy as a side chain and evaluated them on MCF-7 for cytotoxicity *via in vitro* by using the MTT assay thecytotoxic effects of the synthesized analogues were also assessed at the MCF-7 cell line. All the synthesized analogues expressed satisfactory inhibitory action on the tested cell line, using tamoxifen as the standard drug, among which compound **17** was found to be more potent and showed maximum cytotoxicity with an inhibitory effect of 81%.



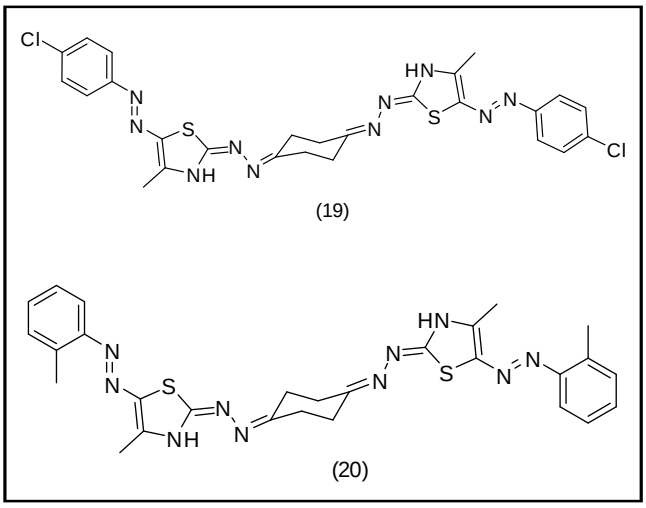
(17)

Mamidala *et al.* (2020) developeda series of novel thiazole compounds by using microwave-assisted multi-component reaction of thiocarbohydrazide, aldehydes with substituted phenacyl bromides. All the targeted thiazole compounds were tested for anticancer action *in vitro* method on various cancer cell lines BT-549, MDA-MB-231/ATCC,MCF-7HS 578T, T-47D and MDA-MB-468 cell lines. Among all the compounds **18** showed themost potent compound. Its cytotoxic effects against metastatic breast 231 cancer cell lines showed 49.4% cells passed away at 50 µg/ml. The docking analysis displayed a good binding affinity with a 6.195 kcal/moldock score.



(18)

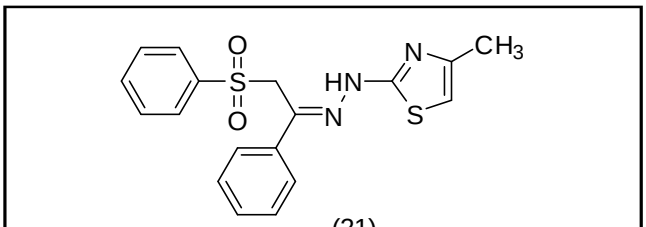
Dawood *et al.* (2021) designed and synthesized different series of bisthiazoles analogues, all the analogues were screened for anticancer activities *in vitro* panel of cancer cell lines. Analogue **19** has shown potentanticancer activities, onthe cervical tumor, Hela cell lines and derivative **20** against the KF-28 cell line. The molecular docking studies were also performed for all the analogues although, both the derivatives have shown higher binding effects and it suggests the inhibition of phosphorylated C-myc.



(19)

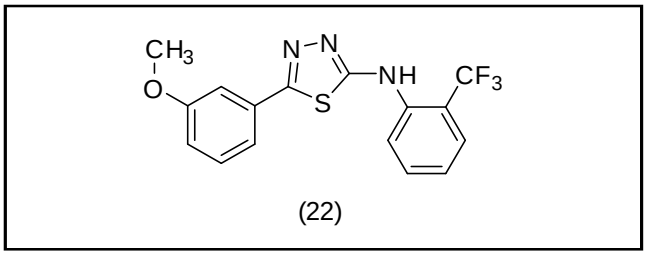
(20)

Farghaly *et al.* (2022) developed and synthesized a series of 1,3-thiazole analogues and also synthesized acetophenone derivatives using substituted phenacyl bromides as a starting product. All the synthesized analogues were tested againstthe multiplication on breast cancer cell lines, *viz.*, HepG2 and MCF-7. The *in vitro* cytotoxic effect, using MTT assay reveals that the 4-methyl thiazole analogue **21**, among all the synthesized derivatives exhibited nine folds more cytotoxicity activity than dasatinibin for MCF-7 cell linewith an IC_{50} of 1.24 ㎜. The docking result also showed that derivative **21** perfectly settle inside the EGFR binding site with a binding score of -7.99.



(21)

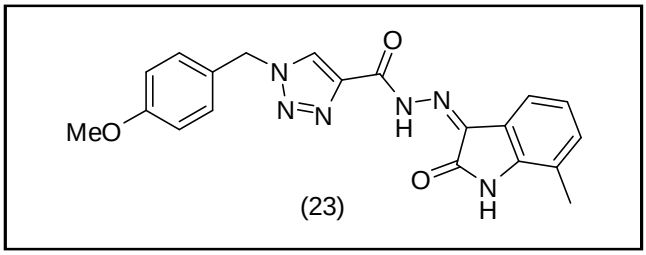
Janowska *et al.* (2022) designed and developed the 1,3,4-thiadiazole analogues and evaluate these analogues for cytotoxic activity using different breast cancer cell lines. The authors report that analogue **22** shows the maximum anticancer activity against MCF-7 having IC_{50} of 49.6 ㎜, etoposide was used as a reference drug. The same was found to be active against MDA-MB-231 cell line, it inhibited the 50% viability of the cells at a concentration of 53.4 ㎜. It also shows the binding affinity of 7.43 kcal/mol for Bax-protein and 7.34 kcal/mol for Caspase 8.



(22)

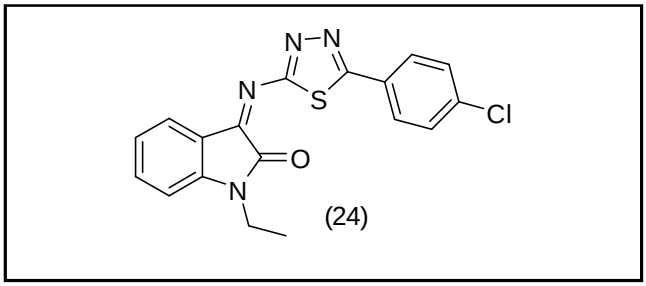
4. Isatin-thiazole hybrid as anticancer agent

Furthermore, some researchers have shown their interest in designing and evaluating the anticancer activity of isatin-thiazole hybrid moieties Aneja *et al.* (2019) designed a set of isatin-triazole hydrazones and tested them for anticancer action on MDA-MB-435s, MCF-7 and HepG2 cells. Authors found that electron donating groups containing isatin-triazole hydrazones derivatives **23** have better binding affinity for microtubule affinity-regulating kinase 4 and enzyme inhibition, as well as inhibition of cell migration with cell proliferation. The MTT assay shows that **23** effectively inhibits the proliferation of three cancer cell lines, *viz.*, MCF-7, MDA-MB-435s and HepG2 with an IC_{50} 6.22, 9.94 and 8.14 ㎜, respectively.



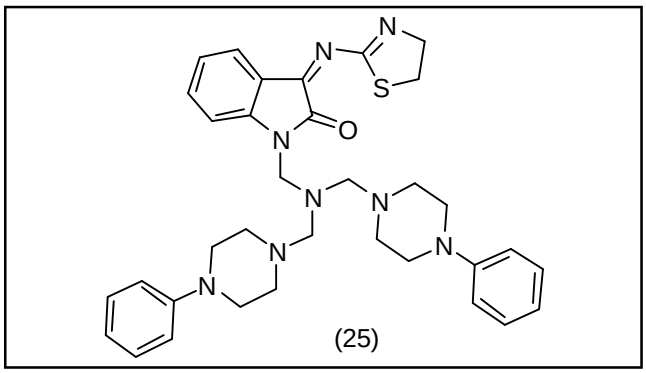
(23)

In another report (Kumar *et al.*, 2017) developed, synthesized and screened the *in vitro* anticancer action against MCF-7 cell lines of novel set of isatin attached hybrid with thiadiazoles. The MTT and SRB assay of synthesized analogues against MCF-7cell lines reveals that chlorobenzene derivative **24** among the entire synthesized derivative shows the most potent cytotoxic activity with IC_{50} 10.46 and 13.04 ㎎/ml for SRB and MTT assay.



(24)

Taher *et al.* (2011) synthesize two novel sets of isatin-thiazoline and isatin-benzimidazole hybrids by reacting isatin mannich base analogues by various aminothiazoline and benzimidazoline further the synthesized series was evaluated for cytotoxicity activity against human breast adenocarcinoma cell line (MCF-7) using sulforhodamine B (SRB) assay taking doxorubicin as standard drug. The study reveals that isatin-linked thiazoline schiff base **25** displayed the highest antibreast cancer activity with IC_{50} of 38.22 ㎜, although, isatin-benzimidazole series also exhibit satisfactory cytotoxicity activity.



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5. Conclusion

Isatin serves as the main nucleus for a variety of cytotoxic and anti-cancer molecules. However, thiazole is a heterocyclic compound found in many anticancer agents due to its versatile nature.This article discusses the anticancer properties of isatin and thiazole molecules. In general, isatin and thiazole moieties are reported to trigger cancer cell death. The rational design of thiazole, isatin, and isatin-containing thiazole hybrids that target a specific receptor/enzyme/protein has led to significant progress in this field. Regarding this, thiazole and isatin analogues are confirmed as agents that inhibit metastasis, inhibitors of microtubule polymerization, inhibitor for protein kinases, apoptotic inducers and tumor suppressors. Lots of these drugs with particular receptors demonstrated strong anticancer effectiveness and selectivity with few toxicity and side effects. Several isatin and thiazole drug candidates with strong effectiveness and a favorable pharmacological profile are in clinical trials. Furthermore, data from preclinical and clinical trials are critically needed to identify the benefits of these novel molecules during the therapeutic development process.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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Neuroprotective effect of *Citrus limon* juice against scopolamine induced amnesia in Wistar rats: Role of cholinergic neurotransmission monitoring and beta-actin signaling

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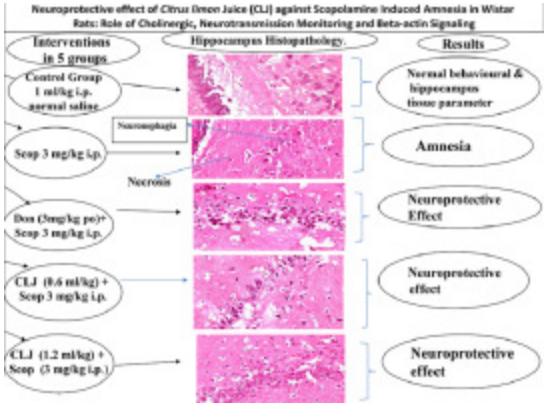
Abstract

Lemon (*Citrus limon*) *Rutaceae) is* one of the most popular world fruit crops, contains active phytochemicals that are polyphenolic compounds, flavonoids, vitamin C, folic acid, potassium, pectin etc. The bioactives compound of lemon are preventing radical-mediated diseases like cancer, diabetes, neurodegenerative disorders but no one reported that effect of

Citrus limon juice on scopolamine induced amnesia model in animal. Rats were divided into five groups**.** Group 1 rats (normal control) received 1 ml/kg normal saline for 14 days intraperitoneally (i.p.). Group 2 rats received scopolamine (3 mg/kg, i.p.) 30 min prior to the trial on the 14th day. Group 3 rats received 3 mg/kg/day of donepezil as pre-treatment for 14 days and scopolamine (3 mg/kg, i.p.) 30 min prior to the trial on the 14^{th} day. Group 4 and 5 rats received *Citrus limon* juice (0.6, 1.2 ml/kg/day) for 14 days and scopolamine (3 mg/kg, i.p) 30 min prior to the trial on 14^{th} day respectively. On the day 14, working memory and long-term memory in rats were tested by experimental paradigms and Morris Water Maze (MWM) task. On day 15, 6 rats of each group were sacrificed and brain tissue of these rats was isolated for the estimation of biochemical parameters, histopathological examination and western blot analysis. Scopolamine treated rats showed significantly increased escape latency ($p<0.0001$), Acetylcholinesterase (AchE) activity ($P < 0.001$), lipid peroxidation ($p<0.001$) and significantly decreased GSH level ($p< 0.001$) in the hippocampus compared to the control group. These parameters were significantly recovered in the rats pretreated with *Citrus limon* juice and donepezil. Histopathological examination showed that *Citrus limon* juice and donepezil provided significant protection against pyramidal cell degeneration, neuronophagia, and thickened endothelium of blood vessels with perivascular and moderately inflammatory cells like eosinophils in scopolamine treated rats. Furthermore, *Citrus limon* juice was found to significantly upregulate the expression levels of ERK and beta-actin proteins in rat hippocampus. The findings of this study demonstrated that *Citrus limon* juice and their constituents have potential to improve the cognitive performance in scopolamine induced amnesia in Wistar rats. Further extensive studies are needed to explore the efficacy of *Citrus limon* juice and its constituents for treatment of various neurodegenerative diseases such as Alzheimer's disease.

Graphical abstract



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Keywords

Citrus limon juice; Antioxidant; Scopolamine; Amnesia; Cholinergic neurotransmission

1. Introduction

According to Chinese Nutrition and Traditional Chinese Medicine (TCM) Lemon *(Citrus limon)* is known for its ability to promote qi circulation, regulate blood circulation, clear heat, eradicate toxins, and resolve phlegm. In general, the ancient Chinese medical texts cite that it enters the Kidney, Liver, Lung, Gallbladder, and Spleen. The flavor of *Citrus limon* is sour, and it is considered to be cold in temperature. *Citrus limon* juice (CLJ) *is* containing bioactive active such as polyphenolic compounds, flavonoids, vitamin C, folic acid, potassium and pectin which are known for their potential against various life-threatening diseases [1,2,3,4]. Polyphenolic compounds have significant potential in preventing and treating free radical-

mediated diseases such as cancer [5], diabetes [6], neurodegenerative disorders [7], process of ageing [8] etc. Viana et al. (2016) reported anxiolytic effect of *Citrus limon* essential oil in animals [9]. Further a study by Lopes et al. (2011) showed the sedative, anxiolytic, and antidepressant activities of *Citrus limon* essential oil in mice [10]. Sarfaraz et al. (2015) conducted a comparative evaluation of anxiolytic effects of pure lemon juice versus reconstituted lemon drink. They found that the anxiolytic effect of lemon was due to its chemical constituent's such as essential oils and flavonoids [11]. Riaz et al. (2014) explored the memory boosting effect of *Citrus limon*, Pomegranate and their combinations in the normal mice [12]. Elham et al. (2018) evaluated the neuroprotective effect of hesperetin and nano-hesperetin on the recognition memory impairment and elevated oxidative stress in a rat model of Alzheimer's disease [13].

Scopolamine (Scop) is a non-selective, competitive, post-synaptic muscarinic receptor inhibitor and can cause cognitive impairment in rodents and humans. It acts by decreasing the effect of acetylcholine in the central nervous system in animals and humans [14, 15]. Scopolamine induced amnesia in rats is a well-established and validated pharmacological model which can be used for screening behavioral tests in animals [16]. Most of the published data has reported the sedative, anxiolytic, and antidepressant activities and memory boosting effect of *Citrus limon* in normal animals. None of the studies to the best of our knowledge have reported the efficacy of CLJ in experimentally induced rat amnesia model. Keeping this in view, the present study was designed to investigate the effect of CLJ on scopolamine induced amnesia in rats.

2. Materials and methods

Citrus limon (purchased from Saifai market, Etawah UP) Acetylcholinesterase Activity Assay Kit (Sigma Aldrich chemicals Pvt. Ltd. India), Scopolamine Hydrobromide (Sigma Aldrich chemicals Pvt. Ltd. India), Ellman's Reagent [5,5- dithio-bis-2-nitrobenzoic acid (Sigma Aldrich chemicals Pvt. Ltd. India), Tricholoacetic acid (Merch chemicals Pvt. Ltd. India), Phosphoric acid (Merch chemicals Pvt. Ltd. India),Thiobarbuturic acids (Merch chemicals Pvt. Ltd. India), Donepezil hydrochloric acid (Aricept), EDTA ((Merch chemicals Pvt. Ltd. India), beta actin (SC 47778, santacruz, USA), ERK (SC 47778, santacruz, USA) and TBST (Merch chemicals Pvt. Ltd. India).

2.1. Extraction of citrus limon juice

Each citrus *limon* was cut into two pieces and its juice was extracted by mechanical pressure using lemon juice machine. The fresh lemon juice was used for animal study. The dose of *citrus limon* juice was selected based on previous reports [17].

2.2. Animal

All the experimental animals were kept in a 12 h light/dark cycle, maintained under constant temperature of 25 ± 1°C and humidity (65±10%). Rats were provided water and food ad libidum, however, all the experimental rats were kept on overnight fasting (∼12 hr) prior to the experiment.

All the animal experiments were performed in accordance with the regulations and guidelines of "committee for the Purpose of Control and Supervision of Experiments on animals (CPCSEA)". This study was approved by the Institutional Animal Ethical committee (IAEC/01/AH/2019-2020) of the UNIVERSITY of Medical Sciences, Saifai, Etawah, UP, India.

36 Wistar either sex rats, age 10-12 week and wt. 100-150 g were used in this study. The rats were divided into five groups each consisting of six rats (n=6) and the duration of the study was study was 15 days.

Group 1 (normal control) received 1 ml/kg normal saline for 14 days via i.p. route. Group 2 received scopolamine (3 mg/kg, i.p) 30 min prior to the trial on the 14th day [18,19]. Group 3 received donepezil 3 mg/kg/day for 14 days and scopolamine (3 mg/kg i.p) 30 min prior to the trial on the 14thday.Groups 4 and 5 received 0.6 ml/kg/day po-CLJ (C1) and 1.2 ml/kg/day po CLJ (C2) for 14 days and scopolamine (3 mg/kg i.p) 30 min prior to the trial on 14th day respectively.

On the day 14, working memory and long-term memory in rats was tested by experimental paradigms like Cook's pole climbing apparatus, Morris Water Maze, and on the 15th day, 6 rats of each group were sacrificed and brain tissues were isolated to estimate the acetyl cholinesterase enzyme (AchE) and brain oxidative stress markers such as lipid peroxidase (LPO), glutathione (GSH) (reduced). The hippocampus of rat brains was dissected for histopathological examination and western blot analysis.

2.3. Behavioural assessment (non-invasive procedures)

2.3.1. Cook's pole climbing apparatus task

This apparatus was used to study the cognitive function, mostly a response to conditioned stimuli during learning & its retention. The rats were allowed to explore the chamber for 45 seconds. Conditioned stimulus (CS), i.e., buzzer signal (bip sound) was turned on and an unconditioned stimulus (US), i.e., electric shock was delivered through the grid floor for 5 sec. Rats learned to associate the buzzer with the impending foot shock and could avoid the foot shock by climbing the pole after the buzzer signal. The time taken by the rats to climb the pole was defined as escape latency. This time was recorded in seconds to study the cognitive function of rats [20, 21].

2.3.2. Morris water maze

The Morris water maze contracted 1.50 m across a circular pool and was 0.60 m high filled with water. Titanium dioxide was added to make the water opaque. A 28×10 cm rectangular escape platform was constructed in water to allow the animal to remain on the top, when it was submerged. The platform submerged 2 cm below the level of the water surface and the temperature of water was maintained at 26 ±2°C. Rats were placed in water from a side of Morris water maze and the time in which rats reached the platform was recorded. Rats were given three trials per day for developing learning and memory. The time taken by the rats to reach the platform was termed as escape latency time [22].

2.4. Biochemical Assay

Animals were sacrificed using ketamine hydrochloride anesthesia. The whole brain was carefully removed from the skull and weighed. 10% w/v brain homogenate was then prepared by homogenizing it in ice-chilled phosphate buffer (pH 8, 0.1M). The homogenate was subsequently centrifuged using a refrigerated centrifuge at 3000 rpm for 10 min, and the supernatant was separated and was used for the biochemical estimation

2.4.1. Determination of Acetylcholinesterase (AchE) activity in hippocampus

AchE was estimated in hippocampus of the rat's brain by using commercially available Acetylcholinesterase activity assay kit (Sigma Aldrich chemicals Pvt. Ltd. India). 10% w/v hippocampus was homogenized in 0.1 M phosphate buffer, pH 7.5, further it was centrifuged at 14 000 rpm at 4 °C for 5 min and was collected supernatants for assay. 10 µl of hippocampus supernatants was added in duplicate to a 96-well plate. Then, 190 µl of the freshly prepared working reagent was added to all sample wells and the plate was tapped to mix. Samples were incubated at room temperature for 2 min and the initial

absorbance at 412 nm was taken followed 10 min later by another reading on same absorbance. The calibrator (200 U/l) given in the Kit that was used as a standard. The result of AChE activity has been represented in units/L [23].

2.4.2. Determine of lipid peroxidation in rat brain

10 % w/v homogenate of hippocampus was mixed with sodium dodecyl sulphate, acetate buffer (pH 3.5), and aqueous solution of thiobarbituric acid. Further its heating at 95°C for 60 min then the red pigment produced. It was extracted withn-butanol-pyridine mixture and was measured absorbance at 532nm., and tetra-methoxy-propane was used as a standard. Lipid peroxidation was determined by estimation of malondialdehyde (MDA) levels expressed as nano moles of MDA/mg of protein [24].

2.4.3. Determine of reduced glutathione

GSH level was measured by the method of Ellman and Lysko. 10% (w/v) homogenate of hippocampus was added in 5% trichloroacetic acid solution. It was centrifuged at 3500 rpm for 10 min. 50 μL supernatant was mixed with 0.32 mol/L disodium hydrogen phosphate and 0.04% 5,5-dithiobis 2-nitrobenzoic acid (DTNB) solution. The yellow-colored substance formed by the reaction of GSH and DTNB was measured at 412 nm. GSH level was expressed in μ moles/mg of tissue. [25].

2.5. Western blotting

The hippocampus tissue of rat brain was homogenized in RIPA lysis buffer. Total protein concentration was assayed by the Bradford method [26]. SDS-PAGE analysis was performed according to the principles of Laemmle with slight modifications [27]. A detailed methodology for the same has been described by us previously [28]. The blots were incubated overnight with primary antibody against phosphor ERK1/2/ ERK ½ and beta-actin in 4°C. The standard reference was β-actin (MA5-15739-HRP). The membrane was washed thrice with TBST and further incubated at room temperature for 3 h with the corresponding beta-actin (SC 47778, Santa Cruz, USA) HRP conjugated secondary antibody (1:2000 dilution). After a 3 TBST washes, the membranes were developed using an enhanced chemiluminescence substrate (Western Bright ECL HRP substrate, Advansta, Melanopark, California, US) in a gel dock system. The quantification of protein was done through densitometric digital analysis of protein bands using Image J software.

2.6. Histopathological Studies

Rat's brains were collected after sacrifice and were fixed in 10% neutral buffered formalin. Subsequently, brain tissues were kept in 10% neutral buffer at 48°C. Then, the brains were routinely embedded in paraffin and stained with hematoxylin eosin. The hippocampal lesions were assessed microscopically at×40 magnifications [29].

2.7. Statistical analysis

Results have been expressed as mean ± SEM. Statistical Analyses was performed with one-way analysis of variance (ANOVA) followed by Dunnett's test. P value less than <0.05 was considered statistically significant.

3. Results

3.1. Effect of CLJ on memory of rats by Cook's pole climbing Apparatus behavior screening

Escape latency was significantly ($p<0.001$) increased in the scopolamine treated group compared to the control group. This decreased significantly ($p<0.001$) in rats pre-treated with donepezil and CLJ (Fig. 1)

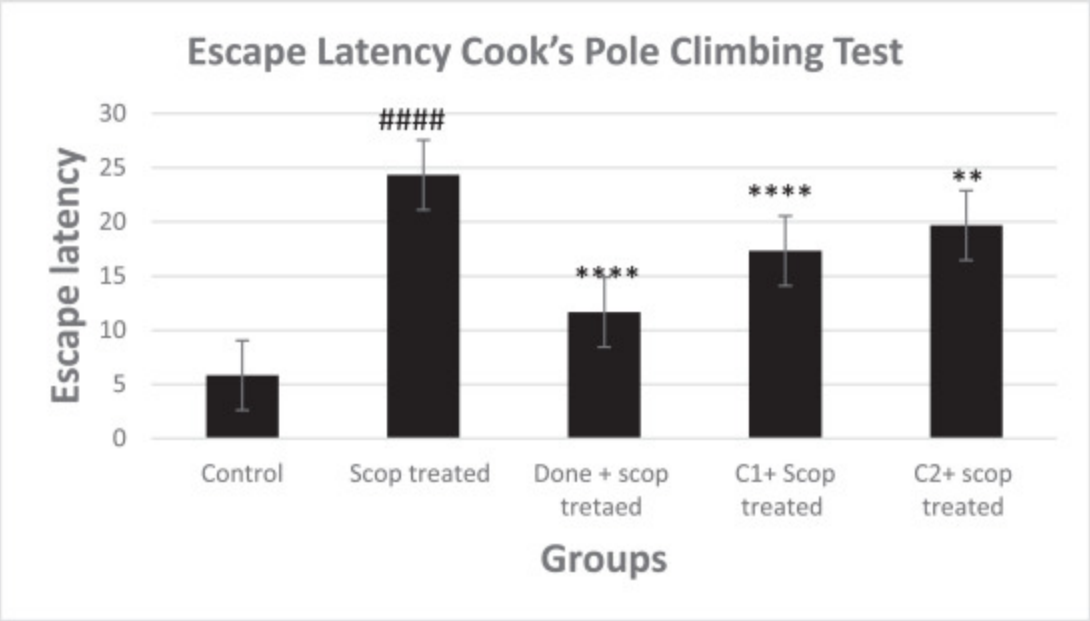

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Fig. 1. Effect of CLJ on escape latency of Cook's Pole Climbing Test in the hippocampus of Scop-treated Rats. Result showed means ± SEM, n=6 in each group. Significant differences ####P < 0.0001 compared to the control group and ****P <0.0001,**P <0.01 compared with the Scop group.

3.2. CLJ improved the long-term spatial memory of scop-treated rats in Morris Water Maze Task (MWT)

Effect of CLJ on spatial memory of the rats was assessed by using MWT. It was found that scopolamine treated rats showed significantly (26.00±2.32 sec; p<0.001) increased escape latency time compared to control group (11.17±2.28 sec) rats. However, this escape latency time significantly decreased, in rats pretreated with donepezil (10.17±1.75 sec; p<0.001), C1 (13.17±1.66 sec; p<0.001) and C2 (17.67±1.22 sec; p<0.05) respectively (Fig.2)

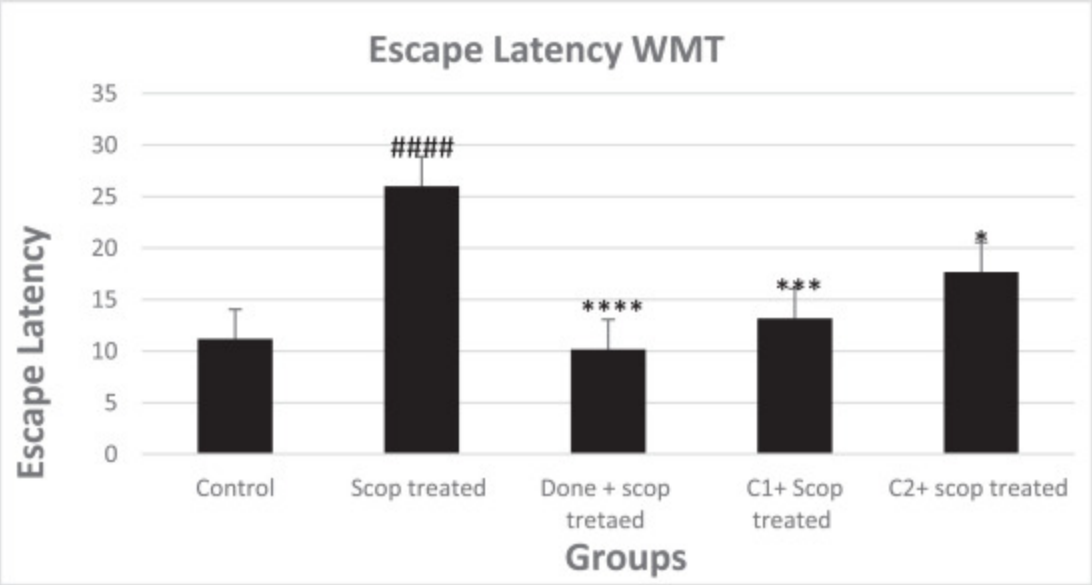

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Fig. 2. Effect of CLJ on escape latency of WMT in the hippocampus of Scop-treated Rats. Result showed means ± SEM, n=6 in each group. Significant differences ####P < 0.0001 compared to the control group and ****P < 0.0001, ***P < 0.001, *P < 0.05, compared with the Scop group.

3.3. Estimation of Acetylcholinesterase (AchE) Enzyme in hippocampus

The result showed that scopolamine treatment induced a significantly increased AchE activity (85.22±10.81; P < 0.001) in the hippocampus compared to the control group (17.60±3.158A significant decrease in the AChE activity was found in the rats pre-treated with donepezil (25.84± 12.6; p< 0.001), C1 (31.78±4.90; p<0.01) and C2 (46.15±11.67; p<0.05) respectively (Fig.3)

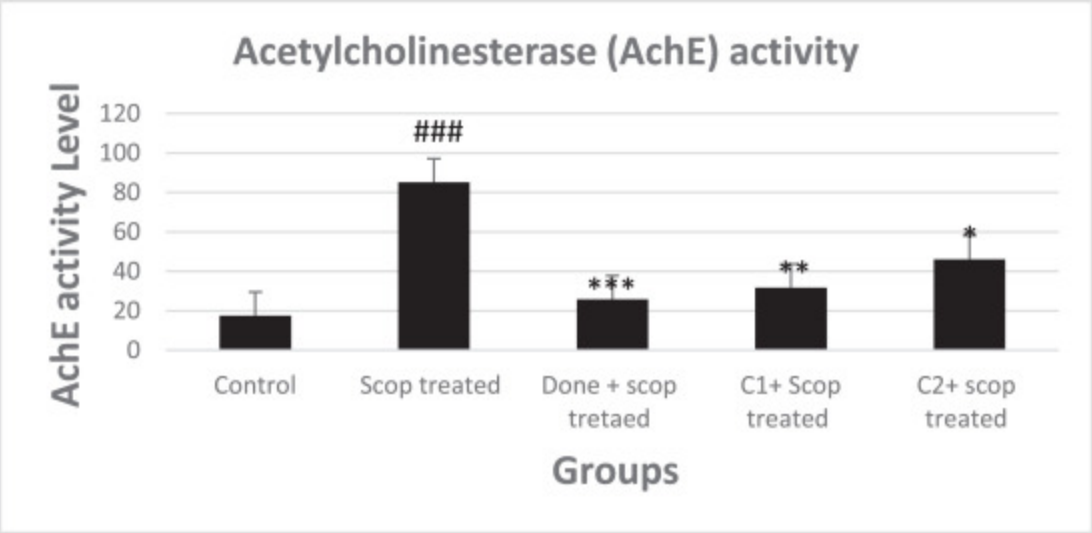

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Fig. 3. Effect of CLJ on acetylcholinesterase (AchE) activity in the hippocampus of Scop-treated Rats. Result showed means ± SEM, n=6 in each group. Significant differences ####P < 0.0001 compared to the control group and ****P < 0.001, **P < 0.01, *P < 0.05, compared with the Scop group.

3.4. Estimation of Reduced Glutathione in the hippocampus of rat's brain

The scopolamine-treated group exhibited significantly decreased GSH level (p< 0.001; 9.92±0.83) compared to the control group (6.24±0.63). Noticeably, donepezil, C1 and C2 treated rats showed significant increase in the level of GSH (p<0.01; 3.87±0.26), (p<0.01;3.49± .039), (p<0.01; 4.15± .022) respectively (Table 1).

Table 1. Effect of CLJ on reduced GSH and MDA level in the hippocampus of Scop-treated Rats.

Groups	Reduced GSH (μ mole /mg of tissue)	Lipid peroxidation (n mole of MDH / mg of tissue)
Normal Control	6.24±0.63	5.05±0.39

Groups	Reduced GSH (μ mole /mg of tissue)	Lipid peroxidation (n mole of MDH / mg of tissue)
Scop treated	1.99±0.21####	9.92±0.83####
Done+scop treated	3.87±0.26**	3.17±0.16****
C1+ scop treated	3.49± .039**	1.222±0.10****
C2+scop treated	4.15+0.22**	2.22±0.15****

Results data Showed means ± SEM, n=6 in each group. Significant differences ####P < 0.0001 and ####P < 0.0001 compared to the control group; **P < 0.01 and ****P < 0.0001 compared with theScop group.

3.5. Estimation of lipid peroxidation in the hippocampus of rat's brain

Scopolamine treated rats showed significantly (p<0.001; 9.92±0.83) increased lipid peroxidase compared to control group (5.05±0.39) which decreased significantly in the rats pretreated with donepezil (p<0.001; 3.17±0.1), C1 (p<0.001; 1.222±0.10), C2 (p<0.001; 2.22±0.15) and hesperetin (P<0.001; 1.17±0.47) group rats respectively (Table1).

3.6. CLJ Promoted the Expression of p-EKR/ERK and Beta action Protein in the Hippocampus of Scopolamine treated rats

Western blot analysis data showed that scopolamine treatment significantly decreased the expression of p-ERK/ERK and beta actin as evident by the significant decrease in the total band volume density (P < 0.0001) compared to the control group (5.8×10^6) However, pre-treatments with CLJ (0.6 ml/kg), and CLJ (1.2 ml /kg) and donepezil (3 mg/kg) significantly recovered p-ERK/ERK(P < 0.001), (P < 0.01), and (P < 0.001) beta actin (P < 0.001) expression which was suppressed by scopolamine. In addition, CLJ (0.6 ml/kg) administration more effectively upregulated the expression of p-ERK/ERK and beta actin compared to the scopolamine treated group (Fig.s4 and b).

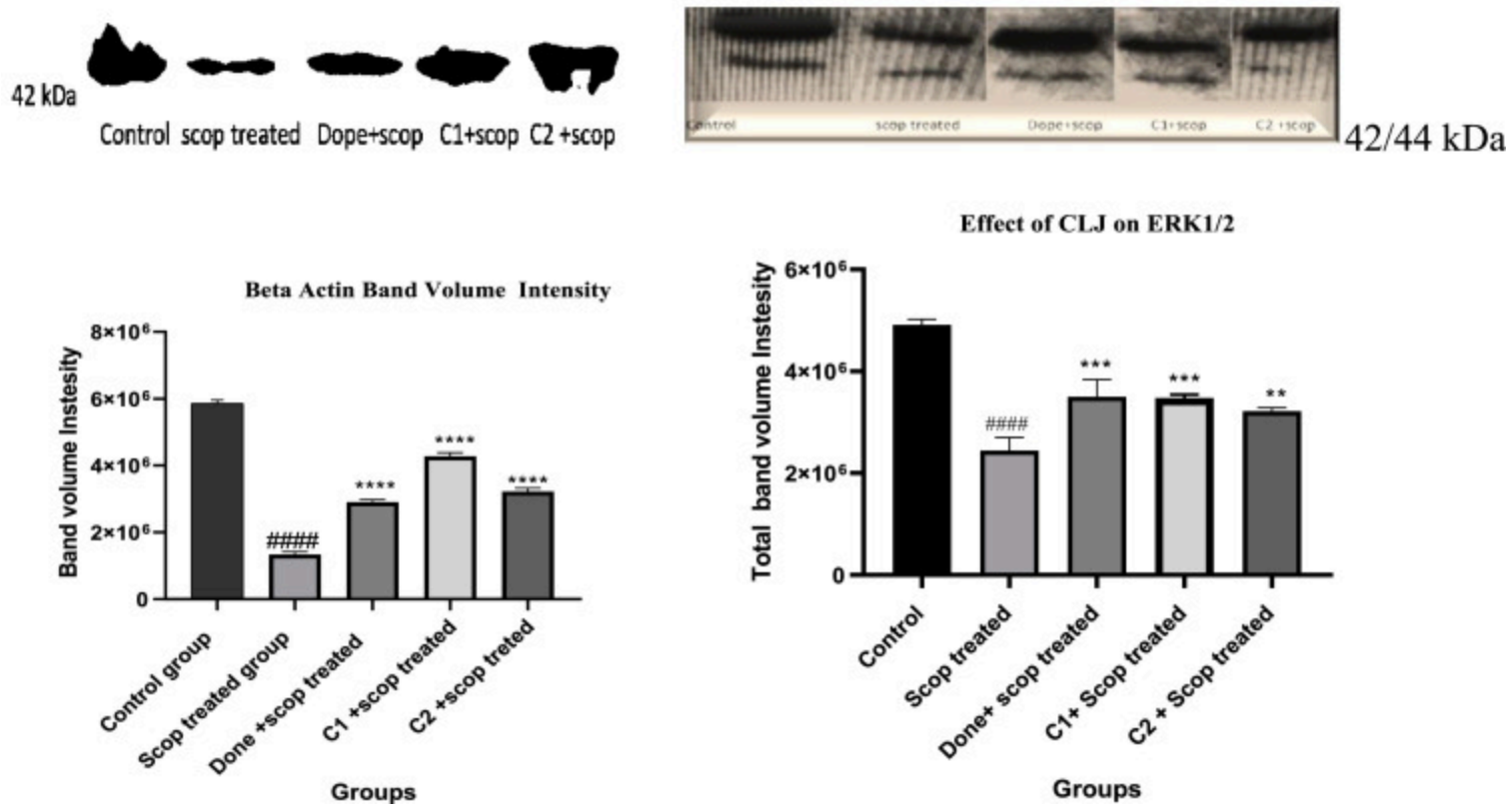

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Fig. 4. Effect of CLJ on the protein expressions of Beta actin in the hippocampus of Scopolamine (scop) treated rats. Data are represented as the means ± SEM, n=5 in each group. Significant differences ####P < 0.0001 compared to the control group; ***P < 0.001, ***P < 0.001, and ***P < 0.001 compared with the Scop group.

3.7. Histopathological examination of hippocampus of rat's brain

The histopathological study of scopolamine treated rats showed a layer of dispersion of granular cells of the dentate gyrus and scattered nerve cells, pyramidal cell degeneration, neuronophagia and thickened endothelium of blood vessels in perivascular and pericellular edema and moderately inflammatory cells like eosinophil were observed (Fig.5). The

histopathology of normal control group rats showed normal CornuAmmonis (CA) areas of hippocampus and dentate gyrus and well-organized glial cells among the neuronal processes. Hippocampus of rats pretreated with donepezil showed significantly restored normal hippocampus and well-organized glial cells among the neuronal processes. However, hippocampus of rats pretreated with C1 and C2 showed normal hippocampus and well-organized glial cells among the neuronal processes, but very minimal inflammatory cells were present, predominantly eosinophils (Fig. 5).

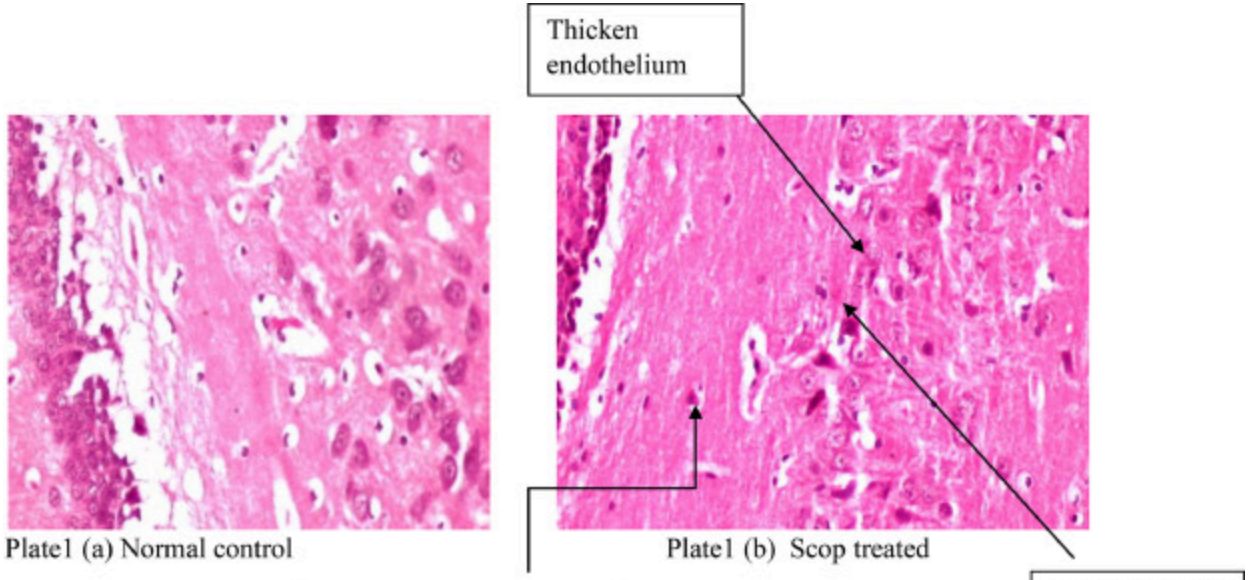

Plate1 (a) Normal control

Plate1 (b) Scop treated

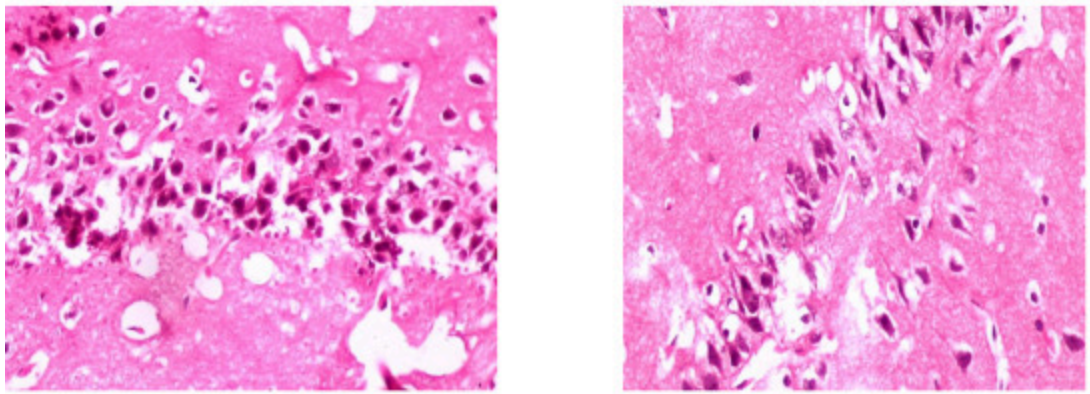

Plate1 (c); Doneperzil + Scop treated

Plate1 (d); C1 + Scop treated

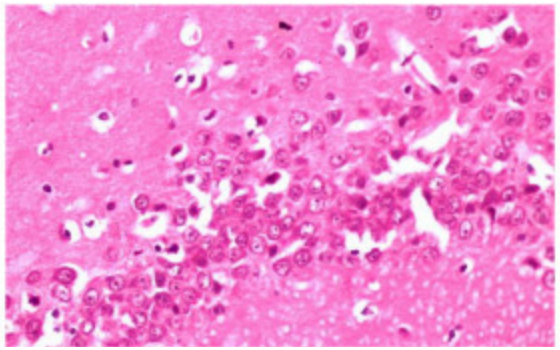

Plate1 (e); C2 + Scop treated

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Fig. 5. Scop treated rat's hippocampus showed scattered nerve cells, pyramidal cells degeneration, neuronophagia and thickened endothelium of blood vessels with moderately inflammatory cells like eosinophil in Plate 1(b) as compared with Normal control Plate 1 (a) which was significantly recovered in pretreated donepezil, C1 and C2 Groups in Plate 1(c), Plate 1(d) and Plate 1 (e) respectively.

4. Discussion

The present study revealed that CLJ has a neuroprotective effect against scopolamine induced amnesia in rats. The results of Cook's pole climbing test and MWM screening indicated that CLJ administration lightened the amnesia which was induced by scopolamine in both Cook's pole climbing test and MWM task. The lightened amnesia may be due to the antioxidant potential of CLJ that improved the cholinergic neurotransmission and enhanced the antioxidant system and activated the β actin signaling.

In the present study, passive avoidance and MWM were utilized to determine the effect of CLJ on scopolamine treated amnesia rats. The Cook's pole climbing test evaluated the long-term memory based on the adverse reinforcement in the rats [30] and the MWM test assessed the long-term, spatial learning aptitude [31].

In Cook's pole climbing test, escape latency was significantly increased in the scopolamine treated group compared to the control group and it was significant decreased in rats pre-treated with donepezil and CLJ (C1 & C2). This indicated that C1 and C2 pre-treatment significantly reduced the loss of memory in scopolamine treated rats.

In MWM test, scopolamine treated rats showed significantly increased escape latency compared to control group rats which indicated that scopolamine impaired spatial learning and long-term memory and these escape latencies were decreased in rats pretreated with donepezil, C1 and C2. However, C1 was found to more significantly the escape latency as compared to C2. This data suggests that C1 more significantly improved the memory of scop-treated rats as compared to C2. The amnesia induced by Scopolamine was reversed by CLJ treatment.

Cognitive functions are regulated by the central cholinergic neurotransmission and various drugs could alter the concentration of cholinergic transmitters that leads to altered performance of memory and learning [32]. Thus, the optimal concentration of neurotransmitters is needed to maintain the normal cognitive function.

The present study showed that scopolamine treatment in rats significantly increased the AchE activity in the hippocampus which was evident by the decreased concentration of acetylcholine because Ache breakdowns into acetyl and choline [33] and it leads to decreased acetylcholine concentration in hippocampus and amnesia is induced in rats. However, rats pre-treated with donepezil, C1 and C2significantly decreased the AChE activity resulting insignificantly increased acetylcholine concentration to improve the cogitative functions.

Rajput and Sarkar (2017) reported that genistein treatment significantly improved the cognitive performance in the diabetic mice by decreasing the AChE activity [34]. A study by et al. (2018) also reported the inhibition of AChE activity and enhanced acetylcholine concentration by genistein administration. It reduced the degradation of acetylcholine and enhanced the cholinergic neurotransmission leading to improved cogitative function [35]. All these data are in coherence to our findings in this study.

Furthermore, scopolamine induced amnesia was also connected with the increased oxidative stress in the hippocampus of the brain which mediates spatial learning and memory [36].

Many previous studies have well documented that oxidative stress is responsible for the pathogenesis of neurodegenerative disorders and natural antioxidants like flavonoids are able to protect against oxidative stress mediated impairment of hippocampus brain cells that leads to cognitive dysfunction [37, 38]. In *citrus limon* juice, flavonoids such as flavanones (eriodictyol, hesperidin, hesperetin, naringin), flavones (apigenin, diosmin), flavanols (quercetin; and their derivatives) have been found which have the capacity to scavenge oxygen-derived free radicals via their anti-oxidant potential [39]. Our

present study showed that scopolamine treated group had significantly decreased GSH level and increased lipid peroxidation (MDA) level in hippocampus of rats which indicated oxidative stress in the hippocampus of scopolamine treated rats. *Citrus limon* juice treatment significantly increased the GSH content and decreased the MDA level in the scopolamine treated rats. This ascertained the antioxidant potential of CLJ. Therefore, based on these results, it could be stated that CLJ can protect against scopolamine induced amnesia mediated by its antioxidant potential and cholinergic neurotransmission in rats.

In addition, histopathological examination of scopolamine treated rats showed cell degeneration, neuronophagia, and thickened endothelium of blood vessels with perivascular and pericellular edema and moderately inflammatory cells like eosinophils. These were significantly restored as normal hippocampus and well-organized glial cells among the neuronal processes in rats Pretreated with donepezil, CLJ (0.6 ml/kg and (1.2 ml/kg) that indicated CLJ protective effect against scopolamine induced hippocampus impairments.

Western blot analysis in various studies have shown that scopolamine induced memory impairments were accompanied with significant inhibition of the ERK expression in the hippocampus [40, 41]. Our present study showed that administration of CLJ up-regulated the expression of ERK in the Scopolamine treated rat's hippocampus. Therefore, CLJ up-regulated the expression of ERK to manage learning and memory against scopolamine induced memory deficit.

Furthermore, scopolamine treatment rats showed significantly decreased beta actin total volume density compared to the control group. However, treatment with CLJ (0.6 ml/kg and 1.2 ml /kg) and donepezil (3 mg/kg) effectively recovered the scopolamine suppressed beta actin expression levels in the hippocampus. Previous study has reported that beta-actin activates endothelial nitric oxide synthase (eNOS), thereby enhancing the NO production [42]. Scopolamine treated rats had significantly decreased beta-actin expression resulting in decreased NO production due to the cerebrovascular endothelial dysfunction and reduced cerebral blood flow leading to learning and memory disorders and cognitive dysfunction which further results in development of dementia [43], [44], [45]. However, pretreated rats showed significantly increased concentration, of beta-actin resulting in, increased production of NO, and increased cerebral blood flow that was responsible for neuroprotective effect of CLJ against scopolamine induced amnesia in rats.

This study has some limitations: A careful literature study revealed no reports establishing the neuroprotective effect of *Citrus limon* juice in an experimentally induced rat amnesia model. Moreover, histopathological investigations and western blot analysis are also not reported so far to assess the neuroprotective effect of *Citrus limon* juice in experimental rat models.

Our study has paved the way for further scientific validation of the efficacy of traditional Chinese medicine like *Citrus limon* juice as a neuroprotective agent. Further, clinical investigations are to be initiated to assess the efficacy of the studied medicinal agent.

5. Conclusion

The study concludes that CLJ has the neuroprotective potential against scopolamine induced amnesia in rats. This neuroprotective effect was mediated by cholinergic neurotransmission, antioxidant system and the ERK and beta actin signaling Mechanism. This molecule if explored further, could be used for treatment against various neurodegenerative diseases such as Alzheimer's disease.

Ethics approval and consent to participate

This was confirmed that animal experiment was performed in accordance with the regulations and guidelines of “committee for the purpose of control and supervision of experiments on animals (CPCSEA)” and institution animal ethical committee (IAEC/01/AH/2019-2020) of the UP University of Medical Sciences, Saifai, Etawah, UP, India has approved study “memory testing on scopolamine induced amnesia in Wistar rats.

Human and animal rights

No humans were used for studies that are the basis of this research. The reported experiments on animals are in accordance with the US National Research Council's "Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Availability of data and materials

The data supporting the findings of the article is available within the article

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Declaration of Cmpeting Interest

The author declares no conflict of interest, financial or otherwise.

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Declared none.

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