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Research and Development in Pharmaceutical Science

Volume I

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PREFACE

Pharmaceutical field encloses a wide number of themens ranging from drug discovery to manufacturing, techniques and technology, regulation and marketing and involves several disciplines such as chemistry, physics, biology biotechnology, pharmaceutics and engineering. The introduction of complex compounds in to medicine resulted in pharmacy becoming a speciazed branch.

To contribute the recent dimensions of the subject, we decided to publish the present book entitled "Research and Development in Pharmaceutical Science (Volume I)". This book is the unified approach to various research areas. The prime goal behind the publication of the book is to bring awareness and exposure to research scholars, academicians and professionals in various topics of current research so that they can actively persue or incorporate the novel methods in their area of work. It is the platform to exchange information and ideas on the recent trends in pharmaceutical sciences and research.

The articles in the book have been contributed by eminent scientists, academicians. Our special thanks and appreciation goes to experts and research workers whose contributions have enriched this book. We thank our publisher Bhumi Publishing, India for taking pains in bringing out the book.

Finally, we will always remain a debtor to all our well-wishers for their blessings, without which this book would not have come into existence.

- Editorial Team

Research and Development in Pharmaceutical Science (Volume I)

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CONTENTS

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SURFACE MODIFIED POLYMERIC NANOPARTICLES FOR ENHANCING DRUG DELIVERY OF METHOTREXATE IN THE BRAIN BY BLOCKING EFFLUX TRANSPORTERS

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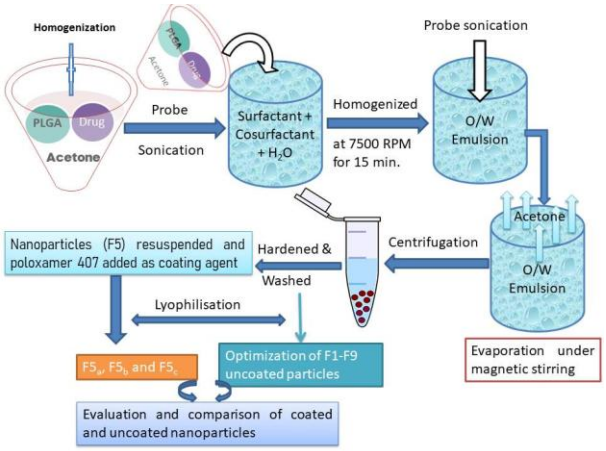
Abstract:

The aim of the present study was to develop, optimize, and evaluate methotrexate containing poloxamer 407 coated polymeric nanoparticles to enhance the concentration of drug in the brain.In the present work methotrexate loaded nanoparticles were formulated by the emulsification solvent evaporation technique. The full 3^2 factorial designs were employed for the determination of polymer and surfactant concentrations effects on different characters, where polymer and surfactant were two variables at three levels. The prepared nanoparticles were optimized and characterized for particle size, polydispersity, zeta potential, entrapment efficiency, scanning electron microscopy, percentage yield, in vitro drug release studies, and sterilization of polymeric nanoparticles.For the delivery of a sufficient amount of methotrexate loaded nanoparticles in the brain, these were coated by using poloxamer 407 (1-2% of total suspension volume). The particle size was observed in the nano range and uniformity of particle size revealed by low PDI value.Thus F5 preparation showed the 192.2 nm particle sizes and its -19.10 mv zeta potential values suggested that it created adequate repulsive forces between the nanoparticles. The percentage entrapment efficiency was observed maximum (72.4%) for the F5 preparation. The percentage yield of methotrexate loaded polymeric nanoparticles was from 58.3 to 76.7% for different preparations (F1-F9). Drug content for different polymeric nanoparticle preparations (F1-F9) was around 72%. The P-values for above-mentioned responses were found ≤ 0.05 which indicated that the model was significant.Our primary findings demonstrate that the designed methotrexate containing PLGA polymeric nanoparticles releasing the methotrexate for a longer period time. The designed preparation could be deliver sufficient amount of methotrexate in the brain. Based on findings, it is possible to exhibit coated polymeric nanoparticles as a good carrier for the increased delivery of methotrexate.

Keywords: Methotrexate, Cancer, Polymeric nanoparticles, Optimization, Poloxamer 407



Introduction:

Nowadays cancer is one of the prime reasons for mortality in the western world. Based on an assessment from 2018, more than 9 million cancer-associated annual mortality and more than 18 million new cancer manifestations will be observed worldwide [1,2]. Prime aims of medicines in today's era should be able to target cells with minimum side effects and significant success rates should be achieved for different treatments [3-5]. To enhance cell death in the case of cancer cells, targeted drug delivery for cancer cells is not sufficient and chemotherapeutic agents must be released at appropriate concentrations to prevent cell growth [6]. Despite that, the progress of medical science to understand advances of cancer diagnosis, treatment and biology, the death rate due to cancer has not decreased significantly in recent years [7, 8]. It is very important in therapeutics of cancer, where the therapy of cancer can show serious consequences on the patient's health, specifically in the manifestation of metastatic cancer where the attack of cancerous cells towards fit organs is the prime reason for cancer-associated mortality [9, 10].

The blood-brain barrier (BBB) is generally composed as a triad of capillary pericytes, astrocytes, and endothelial foot processes [11]. The delivery of drugs is hindered due to BBB and only a few medicaments can reach the brain tissues. That's why many medicaments show good *in-vitro* efficacy but fail to show good *in-vivo* activity. Drug delivery to target the brain is always a tough task unless the existing medicaments have been customized [12]. Small-sized molecules can usually cross BBB inappropriate concentration if the molecular weight of medicaments is less than 400-500 Daltons and 8-10 hydrogen bonds are formed by medicaments when a solvent medium is water. The penetration of the medicaments beyond the blood-brain barrier does not enhance the bulk to lipid solubility when the molecular mass of medicaments is increased. Blood-brain barrier penetration lowers down to 100-folds as the surface area of the drug is enhanced from 52 A^0 (example - a drug with mm of 200 Daltons) to 105 A^0 (e.g. a drug

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of 450 Daltons). Drug permeation across the biological membrane is not related to drug dissipation through solvent water. In contrast to water, the penetration of a drug through a biological membrane is relying on the volume of thedrug [13].

Methotrexate is a folateanalog and consists of pteridine ring, p-aminobenzoic acid, and glutamic acid. Pteridine ring and p-aminobenzoic acid together form the pteroic acid and the difference in chemical structure between methotrexate and naturally occurring folic acid is the presence of the amino group in 4 positions and the methylation of nitrogen in a position of the pteroyl rest. Permeability glycoprotein (P-glycoprotein) is a very well inspected efflux pump of the multi-drugresistant (MDR) genes subfamily. P-glycoprotein is an energy relianttransporter protein involved in an effluxing a number of drugs molecules and hampers their absorption intra-cellularly. MDR of tumor cells is the main reason for the failure of chemotherapy. Tumor cells consisting of multidrug resistance phenotype are usually linked with overexpression of these drug efflux pumps, called as P-glycoprotein efflux pump [14]. Nanomedicine is a drug delivery system that is made by nanotechnology. Drug delivery is the main concern of nanomedicine [15]. Nanomedicine helps and deal with many current limitations in the prevention, diagnosis, and treatment of cancer [16-18]. The coating of nanoparticles with surfactants (polysorbate 80 and 20, poloxamers 184, 188, 338, 407 and 184 polyoxyethylene, 23-laural ether and poloxamer 908) offers the possibility to alter the body distribution of this carrier system after intravenous injection. Coating with polysorbate 80 not only enhances brain concentration after intravenous injection but also increases the uptake of nanoparticles into cultivated bovine brain blood vessel endothelial cells. The coating with polysorbate 80 may induce endocytic uptake of a particle by the endothelial cells lining the blood vessels in the brain, followed by the delivery of the active agents to brain tissue [19].

Materials and Methods:

The methotrexate sample was obtained from Khandelwal laboratories, Mumbai, India, as a generous gift. Poloxamer 407 was procured from NavpadImpex, Mumbai,India.Poly(D,L-lactide-coglycolide) polymer was procured from Nomisma Healthcare, Vadodara, Gujarat, India. Phosophatidylcholine (soya lecithin) was procured from Amitex Agro Product Private Limited, Indore, Madhya Pradesh, India. Tween 80 was procured from Ramagundam Fertilizers And Chemicals Limited (RFCLLtd.) Delhi, India. Solvents, water and other chemicals were used for analytical grade (chemicals that meet specifications of American Chemical Society (ACS) are called reagent or analytical grade).

Preparations of nanoparticles:

In the present work methotrexate loaded polymeric nanoparticles were formulated by the emulsification solvent evaporation technique [20]. First of all Poly (D,L-lactide-coglycolide) and drug were dissolved in acetone with the help of high-speed homogenizer (TOPLAB Digital High-Speed Homogenizer) at 1500 rpm. Then the resultant was sonicated with the help of probe sonicator for 5 min and gradually it added in water phase containing phosophatidylcholineand Tween 80 (surfactant and cosurfactant), then this solution was homogenized again by a using high-speed homogenizer at 7500 rpm for 15 min. Resulting O/W emulsion was sonicated again by using probe sonicator at 40 amplitude for 10 min in an ice-water bath. Then emulsion was stirred by using a magnetic stirrer at the optimum speed for complete evaporation of acetone. After evaporation of the solvent, the resultant was centrifuged at 15,000 rpm for 30 min. Then the supernatant was examined for unentrapped (free drug) by using UV-VIS spectrophotometer (Shimadzu-1800, Japan) at 303 nm. A cryoprotectant mannitol solution was used for the reconstitution of pellets which were constituted at the bottom. The suspension was incubated for 30 min at 37 °C and finally, the pellets were deep-freezed at 80 °C for 8 hrs and under vacuum pressure (< 50 mTorr) lyophilized at a 40 °C temperature for 48 hrs [21, 22].

Optimization of polymeric nanoparticles by response variables effects (3^2 factorial designs):

The full 3^2 factorial designs were employed for the determination of polymer {Poly(D,L-lactide-coglycolide)} and surfactant (phosophatidylcholine) concentrations effects on different characters, where polymer and surfactant were two variables at three levels (polymer- 100, 500, 1000 and surfactant- 100, 150, 200 %w/w). The prepared polymeric nanoparticles were assessed for responses and the experimental data was collected for comparative study. Based on the comparative study of zeta potential, polydispersity index (PDI), particle size, and % drug release optimum formulation was selected for coating.

Characterization of nanoparticles:

The prepared nanoparticles were optimized and characterized for particle size (PS), polydispersity (PDI), zeta potential (ZP), entrapment efficiency (EE), scanning electron microscopy (SEM), percentage yield, in vitro drug release studies, and sterilization of polymeric nanoparticles.

Sterilization of polymeric nanoparticles:

Sterilization of nanoparticles was achieved by an autoclaving technique which was applied to the formulated polymeric nanoparticles (121 °C for 15 min). The sterilized preparations were then exposed to particle size and stability studies.

Zeta potential, particle size, and polydispersity index:

Zeta potential, particle size, and polydispersity index were measured by zetasizer (Horiba Scientific SZ-100, Japan) by dynamic light scattering technique. Before the estimation samples were sonicated and vortexed for getting optimum results.

Determination of drug entrapment efficiency:

The concentration of drug entrapped per unit weight of polymeric nanoparticles was assessed after separation of free drug/unentrapped drug and polymeric from the water phase. In the present work, the dialysis bag method was employed for separation. For the separation, first of all, preparation was taken into dialysis and allows to dialysing the unentrapped drug until the total release of free drug. Throughout the dialysis process, the medium 0.9% saline solution (NaCl) was changed after every 30 min, to determine the precise concentration of a drug. When there was no free drug concentration detected then it was obvious that formulation had been separated. In the 2 ml of the sample solution, there was 0.1 ml Triton X 100 was added to get a clear solution. The drug content was then determined by UV-VIS spectrophotometer (Shimadzu-1800, Japan) at 303 nm.

$$\textbf{Entrapment efficiency(\%)} = \frac{\textbf{Amount of drug in nanoparticles (mg)}}{\textbf{Initial amount of drug (mg)}} \times \textbf{100}$$

Scanning electron microscopy (SEM):

The morphological and structural behavior of drug-containing polymeric nanoparticles preparations was assessed with the help of a scanning electron microscope (SEM) (JOEL, USA). An appropriate sample of drug-loaded polymeric nanoparticles was mounted on aluminium stubs, employing dual-sided adhesive carbon tape and ruptured with a razor blade [23]. The specimens were sputter-coated with palladium/gold at 14 mA for 120 seconds under argon environment for morphology secondary electron emissive scanning electron microscopy and evaluated for morphological/structural behavior at an acceleration voltage of 15 kV.

Percentage yield:

The polymeric nanoparticles were collected from each batch and weighed accurately. The percentage (%) yield was determined by using the following formula [24]:

$$\textbf{\% Yield} = \frac{\textbf{Weight of nanoparticles obtained}}{\textbf{Total weight of drug \& polymer}} \times \textbf{100}$$

Drug content:

A concentration of drug-containing polymeric nanoparticles (equal to 1 mg) from each batch was added in normal saline 50 ml and continuously stirred for 2 hrs. Then constituted colloidal suspensions were centrifuged at 2500 rpm for 30 min at 22±2 °C. The supernatant was

examined for drug content by determining the absorbance at 303 nm by using a UV-VIS spectrophotometer [25].

***In-vitro* drug release studies:**

In this study 50 mg of drug-containing polymeric nanoparticles was placed in basket type USP dissolution test apparatus having stirring element. Then cellophane membrane was used to cover the basket. The phosphate buffer solution (pH 7.4) 900 ml was employed as a dissolution medium at 37 °C. Polymeric nanoparticles loaded basket was revolved at 100 rpm. A definite concentration (5 ml) as a sample was withdrawn at different time intervals of 1, 2, 3, 4, 5, 6, 8, 12, 24, and 48 hrs. At the same time with the help of pipette 5 ml concentration of phosphate buffer solution (pH 7.4) was replaced. The withdrawn samples were estimated by the UV-VIS spectrophotometer (Shimadzu-1800, Japan) at 303 nm [26].

Coating of polymeric nanoparticles:

All preparations of polymeric nanoparticles (F1-F9) were sized in nanometre range and optimized formulation (F5) was coated by poloxamer 407 for modification of the surface of nanoparticles. The poloxamer was used as a coating agent and coated preparation was used for improving the delivery of drug-loaded polymeric nanoparticles in the brain. The polymeric particle preparation process was the same as previous but after the centrifugation, a coating agent was added in different concentrations as follows; for coating, prepared polymeric nanoparticles were resuspended in phosphate-buffered saline (pH 7.4). Then (1% relative to total suspension volume) poloxamer 407 was added and incubated for 30 min at 37 °C and finally, the pellets were deep-freezed at 80 °C for 8 hrs and under vacuum pressure (< 50 mTorr) lyophilized at a 40 °C temperature for 48 hrs [21, 22].

Characterization and comparison of coated nanoparticles with non-coated;

The prepared coated nanoparticles were optimized and characterized for particle size (PS), polydispersity (PDI), zeta potential (ZP), entrapment efficiency (EE), scanning electron microscopy (SEM), percentage yield, in vitro drug release studies, and sterilization of coated polymeric nanoparticles.

For the determination ofthe kinetics of drug release, the data of drug release were fitted in Higuchi, Korsmeyer-Peppas, Hixon-Crowell, First-order and Zero-order kinetic models. Then the values of correlation coefficient were calculated from the linearity of the curves [27].

Statistical analysis:

One way analysis of variance (ANOVA) was used for statistical analysis of data collected from evaluated formulations.

Stability Studies:

Stability studies of formulated polymeric nanoparticles were conducted, by storing optimized formulation at 4±1°C, 30± 2°C, and 45± 2°C in a chamber of stability for 90 days. The samples were examined for particle size and drug content {ICH Q1A (*R*2) 2003} [28].

Results:

Preparations of nanoparticles:

Methotrexate containing polymeric nanoparticles was formulated by the emulsification solvent evaporation technique; a total of nine preparations of drug-loaded nanoparticles were prepared and subjected for assessment for various parameters. The consistency and uniformity of all uncoated formulations were evaluated. The formulated polymeric nanoparticles found odorless, slightly yellowish colour, and showing good consistency.

Optimization of methotrexate loaded polymeric nanoparticles by 3^2 factorial designs:

The purpose of the 3^2 factorial design (3^2- 2 variables at three levels) was to select the stages of different independent variables (Table 1) of polymer concentration (R_1) and surfactant concentration (R_2) with zeta potential, polydispersity index (PDI), particle size, and % drug release.

Table 1: Actual unit with coded labels (3^2 factorial designs)

Factors	Level used		
	Level -1 (Low)	**Level 0 (Medium)**	**Level 1 (High)**
Independent variables			
R_1 = Polymer (%w/w)	100	500	1000
R_2 = Surfactant (%w/w)	100	150	200
Dependent variables			
Y_1 = Zeta potential			
Y_2 = Polydispersity index (PDI)			
Y_3 = Particle size			
Y_4 = % Drug release			

Polymeric nanoparticle preparations were optimized by 3^2 factorial designs and on the basis of response surface methodology and other evaluation parameters (Table 3) of polymeric nanoparticle, F5 formulation was selected as optimized preparation.

Fitting of data to the model:

All the responses of drug-loaded polymeric nanoparticles were analysed for nine preparations and fitted to different models by the application of response surface methodology linearity was observed in the best-fitted model (Fig. 1a, 1b, 1c, and 1d).

Table 2: Composition of methotrexate loaded polymeric nanoparticles

Formulation code	Components (% w/w)			
	Methotrexate	Polymer (R_1)	Surfactant (R_2)	Cosurfactant
F1	100	1000 (1)	100 (-1)	50
F2	100	1000 (1)	150 (0)	50
F3	100	1000 (1)	200 (1)	50
F4	100	500 (0)	100 (-1)	50
F5	100	500 (0)	150 (0)	50
F6	100	500 (0)	200 (1)	50
F7	100	100 (-1)	100 (-1)	50
F8	100	100 (-1)	150 (0)	50
F9	100	100 (-1)	200 (1)	50

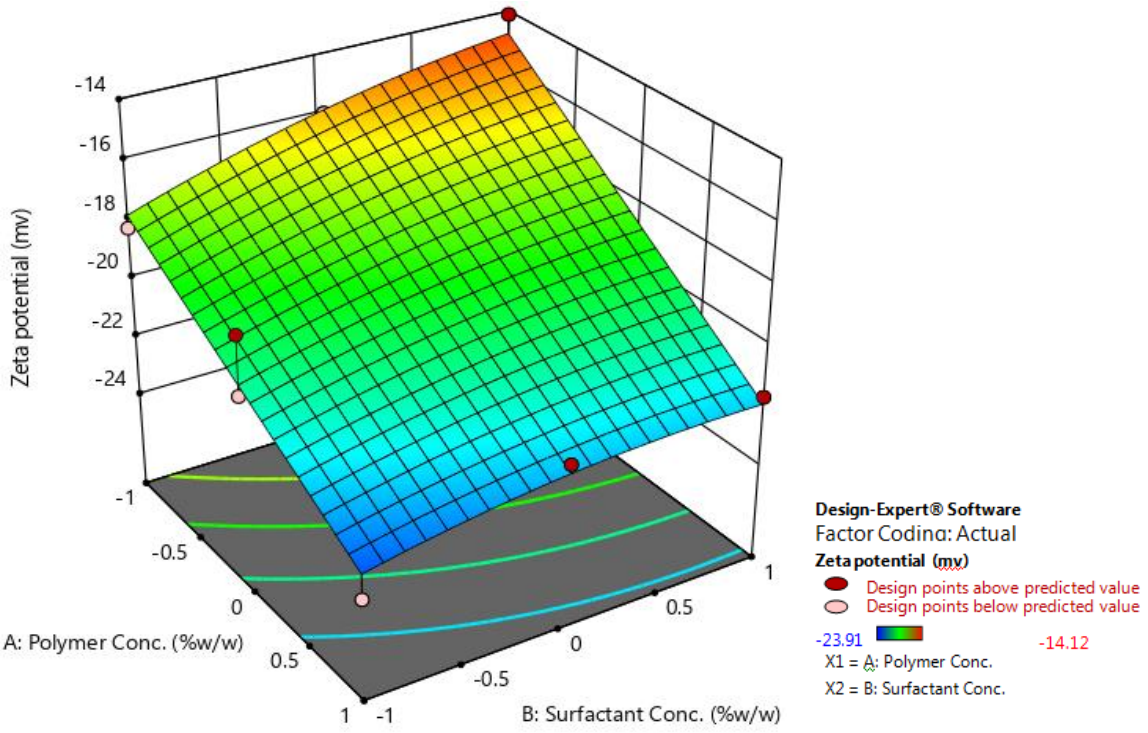

Figure 1: (a) Response surface plot of zeta potential (mv)

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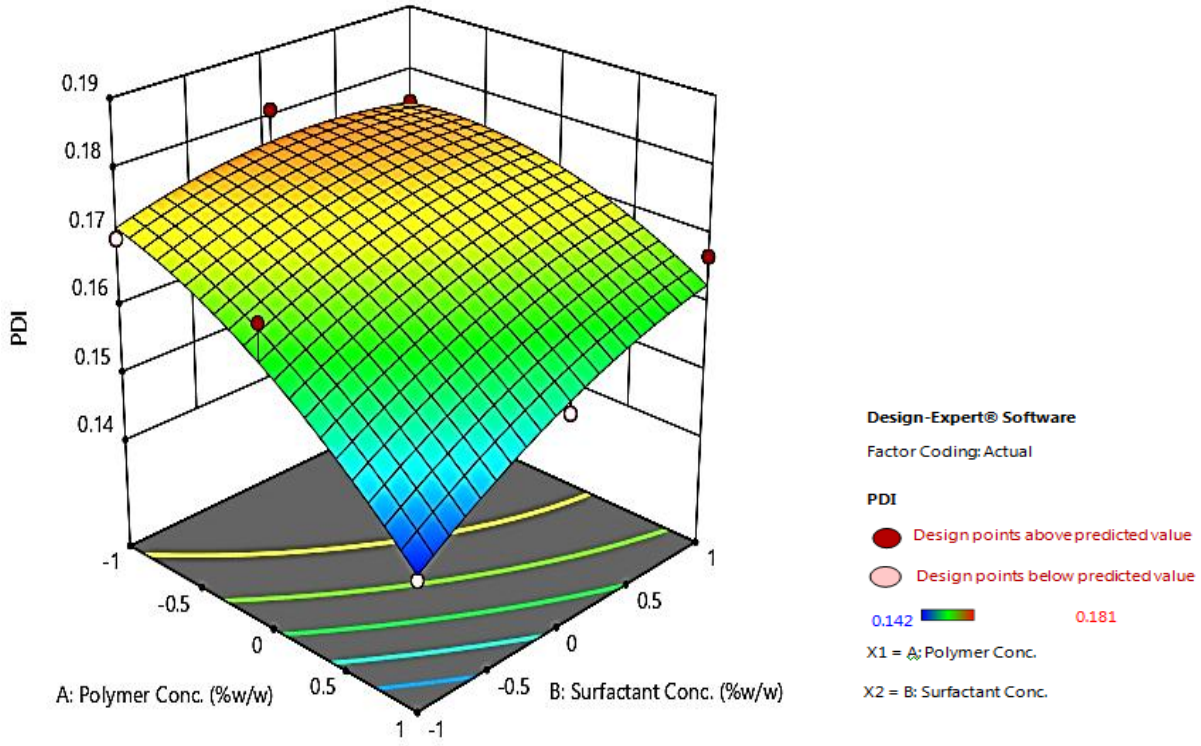

Figure 1: (b) Response surface plot of % poly dispersity index

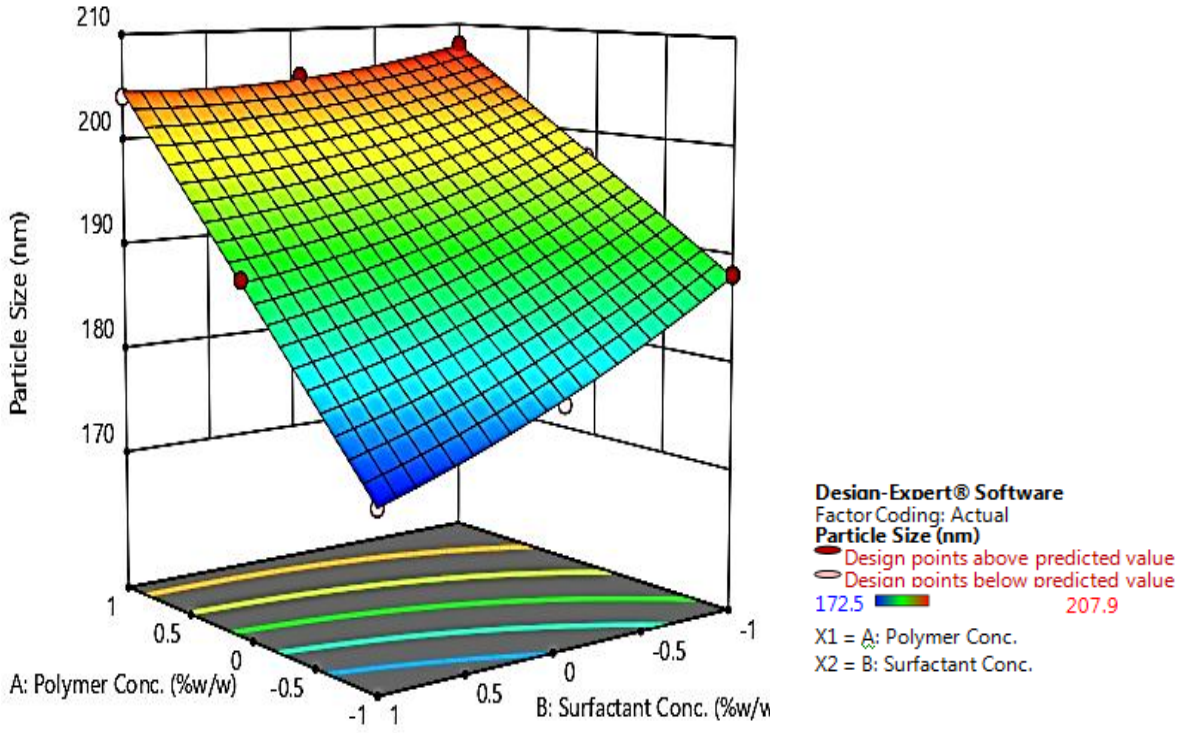

Figure 1: (c) Response surface plot of particle size (nm)

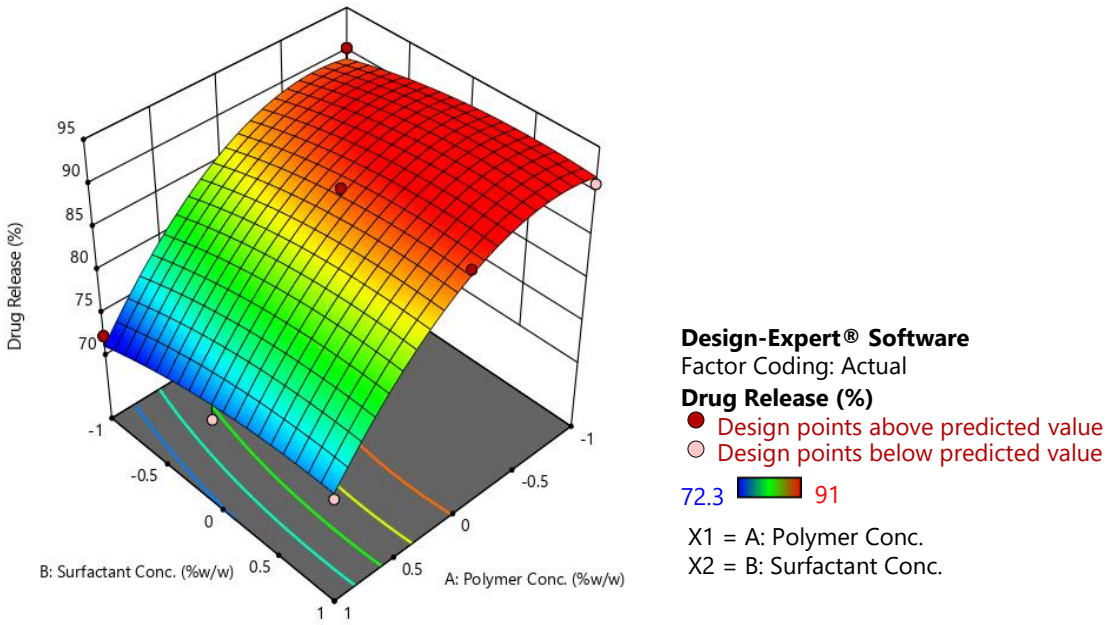

Figure 1: (d) Response surface plot of % drug release

Characterization of nanoemulsion:

Sterility testing:

Sterilization is a primary need of all parenteral formulations. A sterility test was conducted to ensure the sterility of polymeric nanoparticles. Since these particles were administered by using the parenteral route so direct inoculation method was used to conduct the sterility test. In this test, the appropriate concentration of particles was drawn under aseptic conditions from container and separately transferred to 20 ml of thioglycollate medium and 20 ml of soybean-casein digest medium. Then resultant mixture with the medium was incubated for 14 days at 30 °C to 35 °C and 20 °C to 25 °C in case of thioglycolate medium and Soybean-Casein digest medium respectively. The growth of any micro-organism was not detected during the observation.

Zeta potential, particle size, and polydispersity index:

Zeta potential, particle size, and polydispersity index were measured by zetasizer (Horiba Scientific SZ-100, Japan) by dynamic light scattering technique (Fig. 2). Before the estimation samples were sonicated and vortexed for getting optimum results.

Determination of drug entrapment efficiency:

The percentage of entrapment efficiency was found to vary between 51.8±1.11 to 72.4±1.31 %. The percent entrapment efficiency was found to maximum (72.4±1.31 %) for F5 formulation and minimum (51.8±1.11) for F1 formulation. The concentration of polymer and lipid was highest then didn't show significant increment in entrapment efficiency.

Table 3: Characterization of methotrexate loaded polymeric nanoparticles

Formulation code	Particle size (nm)	Zeta potential (mv)	PDI	% EE	% yield	% drug content
F1	207.9±1.1	-23.91±0.4	0.142±0.02	51.8±1.11	58.3±1.24	73.6±1.2
F2	205.3±1.9	-21.80±0.3	0.154±0.01	53.3±1.30	62.1±1.21	70.9±1.1
F3	204.1±1.0	-21.69±0.4	0.167±0.04	57.6±1.11	60.4±1.41	71.3±1.3
F4	197.6±1.4	-20.81±0.5	0.167±0.05	69.8±1.23	70.7±1.36	70.2±0.9
F5	192.2±1.3	-19.10±0.2	0.170±0.02	72.4±1.31	76.6±1.34	76.2±1.1
F6	189.0±1.9	-18.80±0.4	0.171±0.03	68.8±1.46	76.7±1.23	66.1±1.6
F7	188.2±1.1	-18.23±0.5	0.170±0.05	67.4±1.25	74.5±1.26	67.5±0.8
F8	178.6±1.7	-16.00±0.3	0.181±0.06	58.5±1.33	70.9±1.43	72.8±1.2
F9	172.5±1.0	-14.12±0.4	0.175±0.04	59.8±1.21	71.5±1.31	70.0±1.4

Values are shown as mean ± SD (n = 3)

Table 4: *In-vitro* drug release data of prepared polymeric nanoparticles (F1-F9)

Time (h)	% CDR								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
1	7.9±0.37	8.0 ±0.52	8.3±0.38	7.5±0.16	7.7±0.12	8.9±0.18	9.1±0.17	8.4±0.17	9.0±0.14
2	11.0±0.21	11.3±0.46	11.9±0.34	11.1±0.24	12.0±0.25	15.1±0.15	16.3±0.24	16.1±0.24	13.0±0.20
3	14.4±0.26	14.6±0.31	15.4±0.58	14.9±0.30	15.3±0.30	20.9±0.21	23.4±0.36	24.1±0.30	21.5±0.30
4	18.7±0.54	19.1±0.29	19.9±0.44	19.5±0.42	20.0±0.32	28.1±0.41	33.0±0.43	35.4±0.34	30.8±0.33
5	24.6±0.43	23.4±0.34	24.8±0.18	26.0±0.23	28.6±0.29	34.9±0.39	45.2±0.39	46.9±0.40	44.3±0.52
6	29.1±0.11	28.9±0.23	30.1±0.41	32.6±0.53	35.5±0.37	44.5±0.36	57.4±0.55	60.0±0.56	55.7±0.49
8	35.8±0.47	36.7±0.28	38.2±0.29	40.3±0.38	42.3±0.31	58.3±0.41	68.7±0.33	69.4±0.41	66.2±0.36
12	43.3±0.35	44.5±0.19	45.0±0.50	54.3±0.31	57.5±0.46	66.4±0.56	76.1±0.35	78.5±0.35	77.5±0.50
24	61.2±0.28	60.4±0.41	62.8±0.37	69.2±0.48	75.6±0.49	78.2±0.51	89.5±0.52	88.4±0.40	86.4±0.37
48	72.3±0.19	72.5±0.25	74.4±0.36	81.3±0.43	90.1±0.36	90.3±0.19	90.4±0.45	90.9±0.54	91.0±0.59

Values are shown as mean ± SD (n = 3), CDR= Cumulative drug release

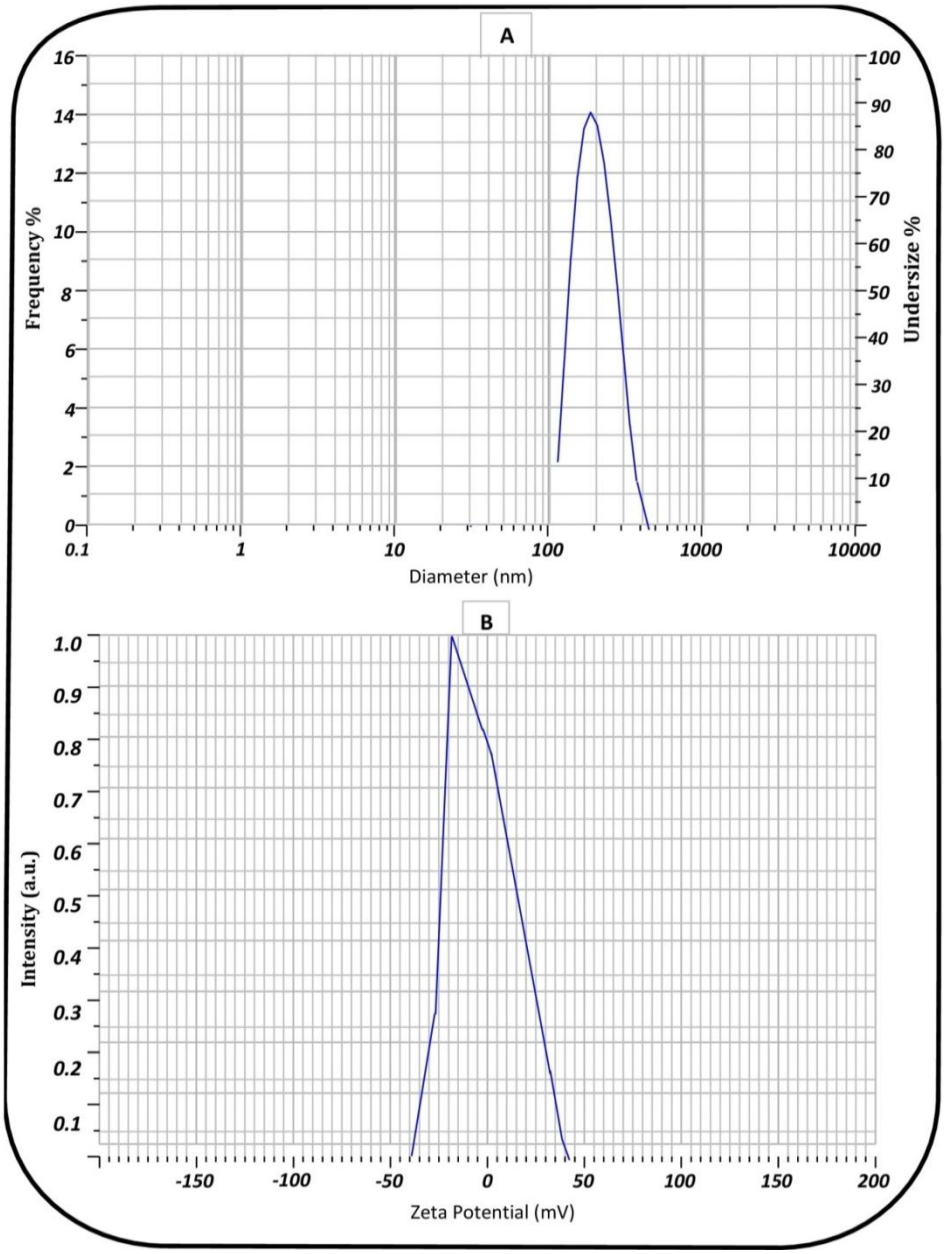

Figure 2: Particle size of optimized formulation (a) and Zeta potential of optimized formulation F5 (b)

Scanning electron microscopy (SEM):

Drug containing polymeric nanoparticles were revealed as small in size with spherical shape (Fig. 3). In the case of methotrexate containing polymeric nanoparticles F5, the particles were found to be aligned. All the nanoparticles were found as discrete entities, showed that aggregation of particles didn't occur during lyophilisation. These polymeric nanoparticles were readily redispersible.

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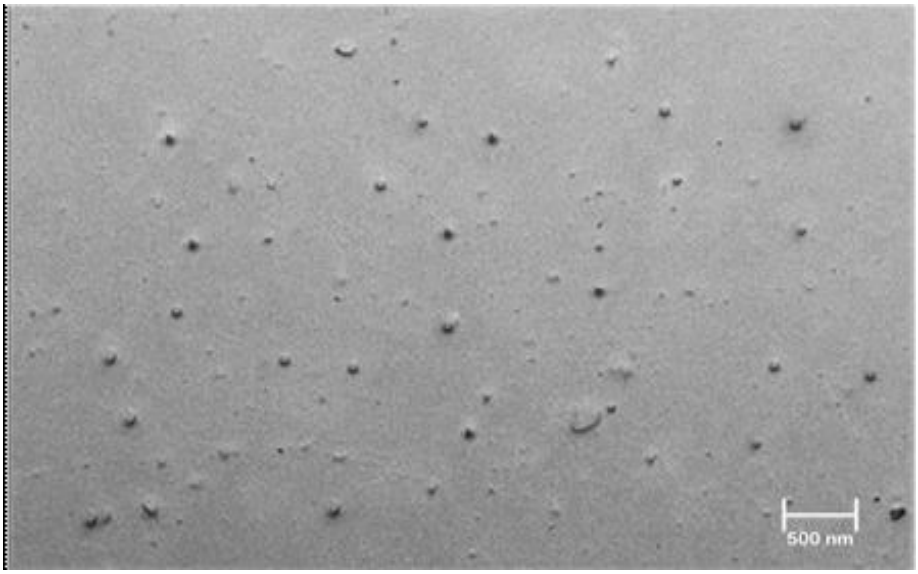

Figure 3: SEM image of methotrexate loaded polymeric nanoparticles formulation (F5)

Percentage yield:

The percentage yield of methotrexate loaded polymeric nanoparticles was from 58.3±1.24 to 76.7±1.23 % for different formulations. The percentage yield of F1, F2, F3, F4, F5, F6, F7, F8, and F9 were observed 58.3±1.24, 62.1±1.21, 60.4±1.41, 70.7±1.36, 76.6±1.34, 76.7±1.23, 74.5±1.26, 70.9±1.43, and 71.5±1.31 respectively (Table 3).

Drug Content:

The drug content of various polymeric nanoparticle preparations (F1-F9) was about 72%. It was observed that when the medium concentration of lipid was employed in formulation then percentage drug content was higher. After that, the percentage drug content was observed to be lesser; this might be due to the capacity of polymer separation.

***In-vitro* drug release studies:**

Results of the *in-vitro* drug release indicate that the preparations showed preliminary burst release and followed by sustained release of the medicaments for a prolonged duration (Table 4). The initial quick-release may be attributed to the little amount of methotrexate on the surface of polymeric nanoparticles. The results of the*in-vitro* drug release revealed that the formulated PLGA polymeric nanoparticles would be able to control drug release for a prolonged period of time. The optimum controlled drug release (90.1±0.36) was found within 48 hrs for F5 preparation. The *in-vitro* drug release rate of methotrexate containing polymeric nanoparticles from F5 formulation is shown in Table 4.

Coating of polymeric nanoparticles:

For delivery of sufficient concentration of methotrexate containing polymeric nanoparticles in the brain, these were coated by using poloxamer 407 (1-2% of total suspension volume). Optimized formulation (F5) was coated by poloxamer 407. In the case of poloxamer 407 (1%) was added directly after the centrifugation process, and it showed that it took more time to coat than other concentration of poloxamer 407. Even some small uncoated particles were observed in the case of a 1% concentration of coating agent. In the case of poloxamer 407 (1.5%), it was coated well and particle size was in range. In the last case of poloxamer 407 (2%), it showed a higher particle size than previous concentrations (Table 5).

Table 5: Composition of coated polymeric nanoparticles (%w/w)

Components (% w/w)	**F5**	**$F5_a$**	**$F5_b$**	**$F5_c$**
Methotrexate	100	100	100	100
Polymer	500	500	500	500
Surfactant	150	150	150	150
Cosurfactant	50	50	50	50
Poloxamer 407 (relative % of total suspension volume)	-	1%	1.5%	2%

F5, $F5_a$, $F5_b$ and $F5_c$ = Different concentrations of coating agent (Poloxamer 407)

Characterization and comparison of coated nanoparticles with non-coated:

Table 6: The different parameters of poloxamer 407 coated nanoparticles

Parameters	**F5**	**$F5_a$**	**$F5_b$**	**$F5_c$**
Particle size (nm)	192.2±1.30	256.5±1.42	270.1±1.85	348.0±1.91
Zeta potential (mv)	-19.10±0.20	-46.0±0.23	-9.5±0.11	-23.0±0.20
% PDI	0.170±0.02	0.312±0.04	0.192±0.02	0.201±0.03
% EE	72.40±1.31	68.59±1.22	76.32±1.26	78.06±1.30
% yield	76.62±1.34	72.2±1.28	80.0±1.40	81.13±1.36
% drug content	76.24±1.10	69.68±1.15	79.31±1.24	80.18±1.80

Values are shown as mean ± SD (n = 3).

Zeta potential, particle size, and polydispersity index of poloxamer 407 coated particles:

The zeta potential of $F5_a$, $F5_b$, and $F5_c$ were found -46.0±0.23, -9.5±0.11, and -23.0±0.20 respectively. The particle size of coated polymeric nanoparticles was observed 256.5±1.42 to 348.0±1.91 nm while optimized uncoated F5 nanoparticles were showed 192.2 nm size. The PDI of $F5_a$, $F5_b$, and $F5_c$ were observed 0.312±0.04, 0.192±0.02, and 0.201±0.03 respectively (Table 6). It was observed that $F5_b$ haspoloxamer 407 (1.5% of total suspension volume) showed optimum zeta potential, particle size, and polydispersity index in all three formulations (Fig. 4).

Scanning electron microscopy (SEM) of poloxamer 407 coated nanoparticles:

The optimized polymeric nanoparticles formulation (F5) coated with poloxamer 407 (1.5% of total suspension volume) and scanning electron microscopy (SEM) of poloxamer 407 coated nanoparticles were revealed as small in size with spherical shape (Fig. 5). These particles were having a large size than F5 formulation. All the coated nanoparticles were found as discrete entities, showed that aggregation of particles didn't occur during lyophilisation.

Determination of % EE, % yield, and drug content:

The % EE of poloxamer 407 coated of polymeric nanoparticles formulations was found to be 68.59±1.22, 76.32±1.26, and 81.13±1.36 % for $F5_a$, $F5_b$, and $F5_c$ respectively. The percentage yield of coated nanoparticles was observed to be 72.2±1.28, 80.0±1.40, and 81.13±1.36 % for $F5_a$, $F5_b$, and $F5_c$ respectively (Table 6). The content of the drug in various coated nanoparticle preparations was found in range 69.68±1.15 to 80.18±1.80%. Although 90% of drug content was observed in F5 nanoparticle preparation. The data of drug content (Table 6) showed that the drug was distributed uniformly throughout the nanoparticle preparations and the drug loss during or after the nanoparticle formulation, it was minimum.

***In-vitro* drug release studies:**

The % drug release data show the sustained release of methotrexate from the optimized nanoparticle formulations. Optimized nanoparticles (F5) and coated nanoparticles formulations ($F5_a$, $F5_b$and $F5_c$) were evaluated for drug release study for 48 hrs (Fig. 6).

An optimized formulation (F5) of coated polymeric nanoparticle's release kinetic mechanism was analyzed through the data fitting to the Peppas model and Higuchi's model and data compared as correlation coefficient (R^2). The correlation coefficient (R^2) for zero-order was observed as 0.9894 which was higher than other models (Table 7). So it was obvious that the release of drug from the formulation followed zero-order release.

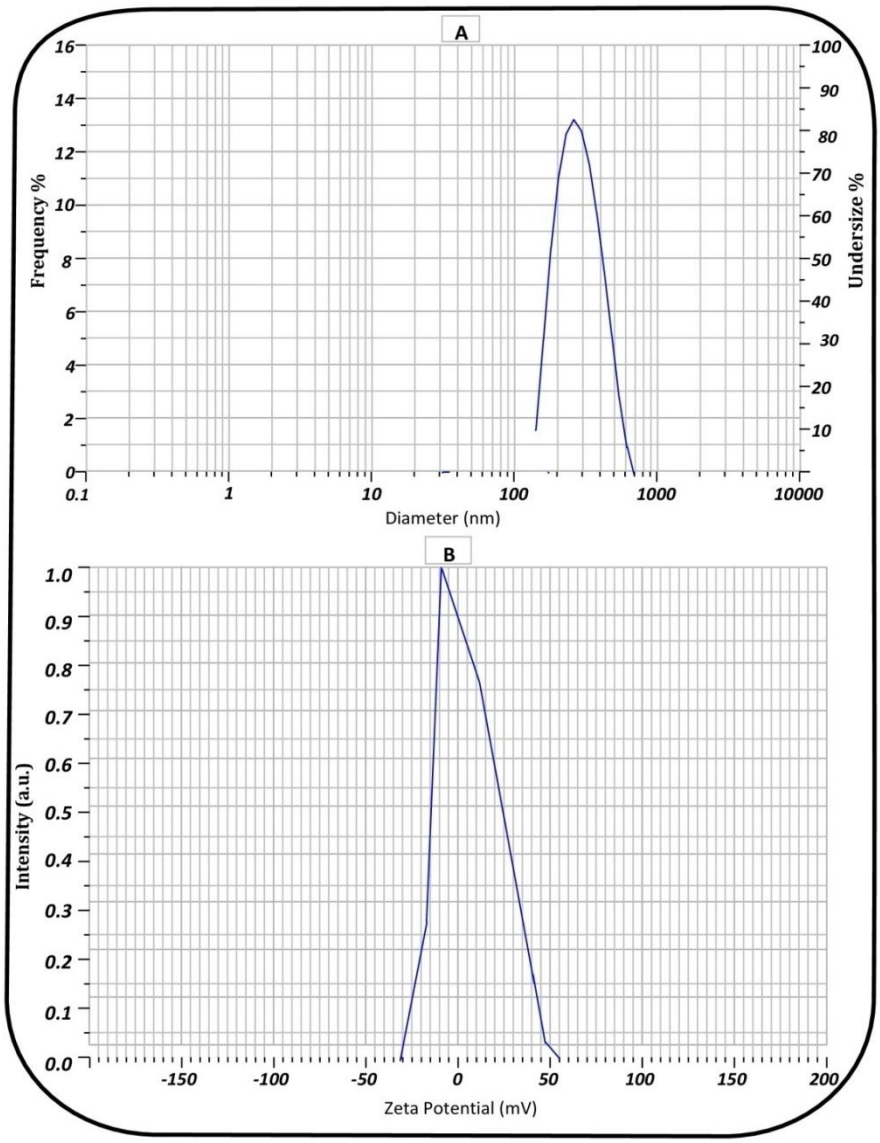

Figure 4: (a) Particle size of optimized coated formulation

(b) Zeta potential of optimized coated formulation ($F5_b$)

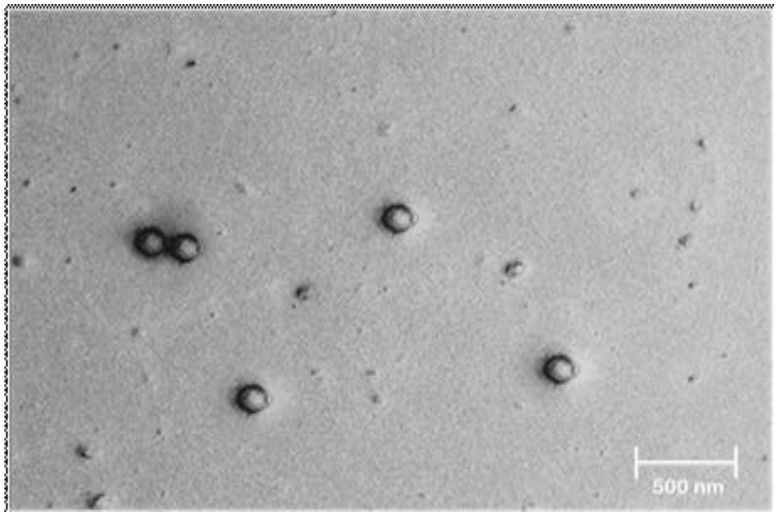

Figure 5: SEM image ofpoloxamer 407 coated polymeric nanoparticles formulation ($F5_b$)

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Table 7: Release kinetic of optimized coated polymeric nanoparticles ($F5_a$, $F5_b$and $F5_c$)

Mechanism of release	Correlation coefficient (R^2)
Zero-order	0.9894
First-order	0.9765
Higuchi's model	0.8892
Hixon-Crowell model	0.9818
Korsmeyer-Peppas model	0.9093

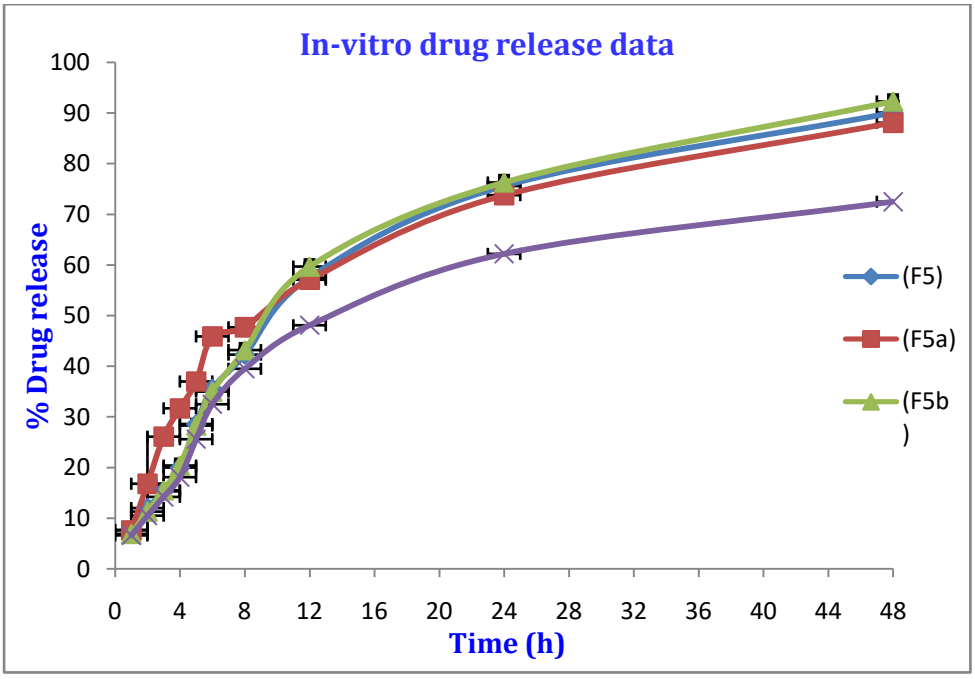

Figure 6: *In-vitro* drug release profile for optimized uncoated particles (F5) and coated nanoparticles formulation ($F5_a$, $F5_b$and $F5_c$)

Statistical analysis:

Table 8: ANOVA forzeta potential, PDI, particle size, and % drug release (F1-F9)

Parameters	Source	Sum of Squares	DF	Mean Square	F-value	p-value	
Zeta potential	Model	87.40	5	17.48	36.11	<0.0001	Significant
PDI	Model	0.0012	5	0.0002	25.77	<0.0001	Significant
Particle Size	Model	1397.37	5	279.47	2494.02	<0.0001	Significant
Drug Release	Model	884.65	5	176.93	43.19	<0.0001	Significant

One way ANOVA method was used for statistical analysis of different formulations. When the P-value ≤ 0.05 than the values of all formulations were observed significant

statistically (Table 8). The **model F-value** implies the model was significant.Linearity was observed for all responses at ≤ 0.05 P-value. So it was obvious that the obtained data was significant.

Table 9: Fit statistics data for zeta potential, PDI, particle size, and %drug release (F1-F9)

Parameters	SD	Mean	CV%	R^2	Adjusted R^2	Predicted R^2	Adequate Precision
Zeta potential	0.6958	-19.20	3.62	0.9475	0.9213	0.7682	19.4736
PDI	0.0031	0.1670	1.84	0.9280	0.8920	0.7065	17.7848
Particle Size	0.3348	192.67	0.1737	0.9992	0.9988	0.9965	172.4947
Drug Release	2.02	85.46	2.37	0.9557	0.9336	0.8736	16.7131

CV = COEFFICIENT OF VARIATION, SD = STANDARD DEVIATION

The **predicted R^2** of showed reasonable agreement with the **adjusted R^2** i.e. the difference is less than 0.2 (Table 9). **Adequate Precision** measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio was greater than 4 and it indicates an adequate signal.

Stability studies:

Table 10: Stability study of optimized coated (F5)and uncoated formulation ($F5_b$)

Time	Temp.	Particle size		Drug content	
		Uncoated SLN	Coated SLN	Uncoated SLN	Coated SLN
Initial	4°C	192.1±1.1	270.2±1.81	77.3±1.2	79.48±1.28
	30°C	192.2±1.3	269.8±1.83	76.5±1.0	80.06±1.20
	45°C	192.6±1.2	270.1±1.87	77.0±1.1	79.90±1.19
1 Months	4°C	192.0±1.2	270.8±1.79	75.9±1.2	78.89±1.23
	30°C	191.8±1.1	271.0±1.80	76.9±0.9	79.36±1.21
	45°C	191.6±1.3	270.6±1.82	76.0±1.1	78.79±1.22
2 Months	4°C	191.7±1.2	270.4±1.90	76.9±1.0	79.98±1.29
	30°C	192.4±1.0	271.2±1.82	75.8±0.9	80.24±1.27
	45°C	191.2±1.1	269.7±1.87	76.1±1.2	79.60±1.22
3 Months	4°C	192.7±1.3	270.4±1.76	75.6±1.0	79.46±1.20
	30°C	191.5±1.1	271.3±1.80	76.4±1.3	78.52±1.18
	45°C	191.6±1.3	269.4±1.78	77.0±1.1	79.01±1.26

Stability studies of formulated polymeric nanoparticles were conducted, by storing optimized formulations (F5 and $F5_b$) at 4±1°C, 30± 2°C, and 45± 2°C in the chamber of stability for 90 days. The samples were examined for particle size and drug content (Table 10).

Discussion:

The poloxamer 407 coated polymeric nanoparticles were used as a carrier for delivery of methotrexate due to its BBB permeation enhancing characteristics. With the help of poloxamer 407 coated nanoparticles delivery of drug, we minimized the problem related to the conventional dosage form of methotrexate. The polymeric nanoparticles preparation was methotrexate containing a multicomponent system having PLGA, lipid/surfactant, and poloxamer 407 as a coating agent. The nanoparticles were prepared by figuring out the range of quantities for all components. All polymeric nanoparticles preparations (F1-F9) were optimized for zeta potential, polydispersity index (PDI), particle size, and % drug release. The particle size was observed in the nano range and uniformity of particle size revealed by low PDI value. Nanoparticles with nano-size have important properties such as enhanced drug delivery, prolonged circulation time in blood, and minimum toxicity. To ensure its parenteral dosage form development sterility test performed and there was no micro-organism detected during the test.Thus F5 preparation showed the 192.2 nm particle sizes and its -19.10 mv zeta potential values suggested that it created adequate repulsive forces between the nanoparticles. The percentage entrapment efficiency was observed maximum (72.4%) for the F5 preparation. The percentage yield of methotrexate loaded polymeric nanoparticles was from 58.3 to 76.7% for different preparations (F1-F9). Drug content for different polymeric nanoparticle preparations (F1-F9) was around 72%. It was found that drug content was higher when lipid concentration was at the medium level (150%w/w).The cumulative percent drug release of drug for different formulations (F1–F9) was observed to be from 72.3 to 91%. Among all preparations, sustain and optimum drug (91.3) was released from F5 preparation. Initial burst release of methotrexate was observed from polymeric nanoparticles for all preparations but the most sustained release was observed from F5 formulation. Initial burst release might be due to the dissolution of methotrexate on the polymeric nanoparticle's surface. The protonated amino residues on methotrexate and the anionic group showed the electrostatic interaction, which was superintendedfor the surface-bound interactions involved in the initial burst release of drug. On findings, we observed the optimum sustained *in-vitro* drug release from F5 formulation.Based on these considerations F5 preparation was selected for poloxamer 407 coating. When zeta potential, % PDI, particle size, and drug release were compared through one-way analysis of variance for all preparations then

P-values found <0.0001 for above-mentioned responses. The P-values for above-mentioned responses were found ≤ 0.05 which indicated that the model was significant [29]. The fit statistics data for zeta potential, PDI, particle size, and %drug release (F1-F9) indicated the significant agreement with the predicted R^2 and adjusted R^2. The difference between the predicted R^2 and adjusted R^2 should be less than 0.2 and in this research work, it was found less than 0.2. So it was clear that the collected data was significant. For delivery of sufficient concentration of methotrexate containing polymeric nanoparticles in the brain, F5 polymeric nanoparticle was selected for poloxamer 407 (1-2% of total suspension volume) coating. In the case of poloxamer 407 (1%), it took more time to coat than other concentrations of poloxamer 407. Even some small uncoated particles were observed in the case of 1% concentration of coating agent. In the case of poloxamer 407 (1.5%), it was coated well and particle size was in range. In the last case of poloxamer 407 (2%), it showed higher particle size than 1% and 1.5% concentrations. The $F5_b$ formulation showed -9.5 mv zeta potential which was adequate to produce repulsive forces between the particles. According to PDI values which were shown in Table 6 indicated that $F5_a$and $F5_c$ didn't show good uniformity than $F5_b$.The low %PDI value (0.192) of $F5_b$ indicated the uniformity of poloxamer 407 coated nanoparticles.Entrapment efficiency, drug content, and % yield showed significant values for the coated polymeric nanoparticles.Morphology and structure of coated and uncoated nanoparticles were confirmed through SEM analysis [30]. These were revealed as spherical in size and showed a slightly rough surface. Zero-order release kinetic was followed by the coated polymeric nanoparticles and it was confirmed by the data fitting in different models. So it was clear that preparation followed zero-order release kinetic. The value of the correlation coefficient for zero-order kinetic was higher than other models. So it was clear that preparation followed the zero-order release. The stability was also checked for uncoated and coated polymeric nanoparticles and during the study,there were no changes were found in particle size and drug content.Coating of poloxamer 407 improves the permeability through BBB and providesa sufficient amount of drug in the brain. Thus methotrexate containing coated nanoparticles could be beneficial to increase the concentration of drug in the brain than the conventional dosage form.

Conclusion:

The poloxamer 407 coated polymeric nanoparticles of methotrexate with optimum drug release was formulated to enhancing BBB permeability. Our primary findings demonstrate that the designed methotrexate containing PLGA polymeric nanoparticles releasing the methotrexate for a longer period of time. The designed preparation could be deliver a sufficient amount of

methotrexate in the brain. On the basis of findings, it is possible to exhibit coated polymeric nanoparticles as a good carrier for the increased delivery of methotrexate.

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ANTIMYCOBACTERIAL, ANTIOXIDANT AND CYTOTOXIC PROFILE OF FOUR INDIAN SPICES

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Abstract:

Multidrug resistant bacilli causing the tuberculosis in human and other mycobacterial diseases are widely distributed in the world. The contemporary antimycobacterials: are not pleasing and do not have good control power due to long time period to alleviate and increasing resistance against present antimycobacterial drugs is a serious concern. For this reason scientists are increasingly turning their attention to herbal products, looking for new lead antimycobacterial drugs. Indian spices have become important due to their potential antimycobacterial and stimulating effects in the animal digestive system. The antimycobacterial effectiveness of spices depend on the composition of spice, and concentration, type and concentrations of the target microorganism, substrate composition, and processing and food storage conditions. Spice plants have been traditionally used for much of the diverse activities. In this study we aimed to explore the antimycobacterial, antioxidant, cytotoxic profiles of four Indian spices with scientific experimental and it was observed that selected spices have an antimycobacterial commotion along with the antioxidant potentials also we found that there is negligible cytotoxicity of the all spices.

Introduction:

Spices are long known for their multipurpose activity, from ancient time it was well known for their use in food preparations and have a unique value in all over the world. There are several reports shown to have a wide variety of potentials of the spices including, antibacterial, anticancer, antidibetic. There are hundreds of the reports showed the growth inhibitory activity against variety of the human pathogens. In case of the diseases, one of the common diseases found in all over the world and has taken billions of life; Tuberculosis; the causative agent is Mycobacterium tuberculosis. A bacilli having prospective to invade the human lungs and also

the whole body, this is the famous for the important issue that it is only who is able to invade and able to grow in the macrophages; the integral part of the human immune system. Since 1980, Rifampicin, ethambutol, isozianid, Pyrazinamide, are worldwide famous antimycobacterial structures used to treat tuberculosis. But later on the bacilli shows the mild resistance against these antibiotics. From this point of observations, scientist moved towards the drugs from plant origin, and found many plant-derived compounds e.g. Berberine, lichoisoflavone, erygibisoflavone, phaseollidin, erythrabyssin II and tryptanthrin as potential antitubercular agents. But the bacterial resistance is increased time to time. In the present study, the spices chosen are used in daily preparations, are cheap, and according to the literature, some of them were not yet tested for antimycobacterials, so this exploration will be helpful in the plant medicine research henceforth able to find novel molecules from plant source.

Material and Methods:

Materials

Three mycobacterial strains used for this study; *Mycobacterium tuberculosis H37Rv, Mycobacterium smegmatis, mycobacterium phlei* are purchased from MTCC IMTECH, Chandigarh. The above cultures are preserved in Middlebrook 7H9 medium and used for assay using Middlebrook 7 H 9 broth medium. The selected spices are *Piper nigrum, Cinnamonum zeylanicum, Elettaria cardamom, Szyzigium aromaticum.* The spices were purchased from local market, and spices were shed dried for 4- 5 days. And used for the extraction purposes by grinding in a grinder.

Solvent extraction of selected plants

The grinding samples were used for the extraction. Fifteen gram of grinding powder of each spice were filled separately in the thimble and extracted successively with 250ml of each water, ethanol, chloroform, methanol for 3-5 hours. All the extracts were concentrated using rotary evaporator. After complete evaporation using rotary evaporator, each of these solvent extract was weight and preserved at 4^0 C in airtight tubes.

Antimycobacterial activity

Sterile Middlebrook agar was poured in to sterile standard Petri plates (20ml). This was then inoculated with 0.1ml of each cultures of, *M. tuberculosis, M. smgematis, M. phlei* (which was previously incubated for 24 in Middlebrook 7 H 9 broth) the spreding were carried out by sterile glass spreader. After spreading cultures on medium, three cups of 7mm diameter were prepared with the help of a sterile borer so that appropriate distance is made in each of well among three wells. The cups were filled by adding 50 µL of the different spices extracts with 50

mg/ml concentration while rifampicin (10µg/ml) used as standard. The plates were incubated for 24 h at 37˚c. After incubation the zones of inhibition around each cup were measured (including cup) in millimeter with the help of zone measuring scale (Himedia Pvt. India).

The resazurin microtiter assay

The inoculum was prepared from fresh Middlebrook medium in 7H9 medium (consisting of Middlebrook 7H9 broth containing 0.1% Casitone and 0.5% glycerol and supplemented with oleic acid, albumin, dextrose, and catalase [OADC]), adjusted to a no. 1 McFarland tube(3.0×10^8 CFU/ml), and diluted 1:20; 100 µl was used as the inoculum. The RIF stock solution was thawed and diluted in 7H9-S medium to four times the highest final concentration tested. Serial twofold dilutions of RIF were prepared directly in a sterile 96-well flat-bottom microtiter plate by using 100 µl of 7H9. The range of concentrations tested for RIF was 8.0 to 0.25 µg/ml. Same set were prepared for the plant extracts containing the concentrations 80 to 0.625 mg/ml. growth control containing no antibiotic and a sterile control without inoculation were also prepared on each plate. Two hundred microliters of sterile water was added to all perimeter wells to avoid evaporation during incubation. The plate was covered with its lid, replaced in the original bag, and incubated at 37°C under a normal atmosphere. After 7 days of incubation, 30 µl of resazurin solution (0.01% in distilled water and filter sterilized) was added to each well and the plate was re-incubated overnight. Then, at day 8, a change in color from blue (oxidized state) to pink (reduced state) indicated the growth of bacteria, and the MIC was defined as the lowest concentration of extract that prevented this change in color.

Evaluation of Antioxidant activity

DPPD radical assay

The DPPH radical scavenging assay was carried out according to a reported method. The reaction mixture contained different concentrations of plant sample (50 mg/ml, in absolute ethanol) and DPPH radical (10^{-4} M in absolute ethanol) solution. The contents of the reaction mixture were observed spectrophotometrically at 517 nm for 20 min. Ascorbic acid (1 mM) was used as a reference compound.

OH radical assay

The scavenging activity for OH radicals was measured with Fenton reaction. Reaction mixture contained 60µl of 1m nM of $Fecl_3$, 90µl of 1mM- 1,10phenanthrolin, 2.4ml of 0.2M phosphate buffer(ph-7.8), 150 µl of 0.17M H_2O_2 and 1.5 ml of sample. Adding H_2O_2 started the reaction after incubation at room temperature for 5mins the absorbance of the mixture at 560 nm was measured with spectrophotometer. Vitamin E (alpha tocopherol) taken as the standard. The results of the assay are summarized in table 3 (14).

Evaluation of Cytotoxicity

MTT assay

The provided cell lines (Chang liver) is directly used for assay within 2 hours in the medium provided by N.C.C.S. Pune. The cytotoxic potential of the Alcoholic beverages was determined following incubation of model cells using the MTT assay. The assay uses a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to assess cellular metabolism and hence viability. In metabolically active cells, MTT is reduced by the mitochondrial enzymes succinate dehydrogenase with the formation of insoluble purple formazan crystals. These are then solubilised, and the absorbance measured spectrophotometrically at 570 nm. Cells were seeded at a density of $4x10^3$ cells/well into a sterile 96-well plate and allowed to adhere overnight. Then 20µl of the appropriate extract solution in the concentration range of 0-100µg/ml was added. Cells were incubated with the extract for a period of 24, 48, 72, or 96 h. Following the required incubation period, 50µl of MTT was added to each well and the plates were incubated at 37^oC in a humid atmosphere with 5% CO_2 and 95% air for 4 h. The media was then gently aspirated, and 150µl DMSO was added to dissolve the formazan crystals. The amount of formazan product was measured spectrophotometrically at 570 nm using a Tunable Thermo made Elisa reader. Each sample concentration had three replicates per assay, and each experiment was carried out on three separate occasions. This value was used as a means for comparing the cytotoxicity of the extract for each of the two cell lines used in this experiment. The results of the assay are summarized in table 2.

Result and Discussion:

Water, ethanol, chloroform, methanol extracts of four plants tested for their antimycobacterial activity against *M. tuberculosis, M. smegmatis, M. phlei*, on agar using of Middlebrook 7 H 11 agar base medium with OAC.

The results are summarized in table 1 with rifampicin used as a standard. Among studied methanolic extracts of *P. nigrum* and *C. xeylanium* and water extract of *S. aromaticum* show highest efficacy at the concentrations of 50mg/ml. all other extracts show some sort of antimycobacterial growth inhibition. But there is comparatively negligible activity of chloroform extract of *C. zeylanicum,* water and ethanolic extract of *E. cardamomum*. In this assay, the methanolic extract of any of the plant shows potent activity against all mycobacterial strains.

Using visual observations of the assay plate, the MICs of the all the extracts for all the three stains were determined and the results were obtained after 8 days of incubation.

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Table 1: Antimycobacterial profile of the four extracts of the selected spices zones in mm

Name of spice	Extract	*M. tuberculosis*	*M. smegmatis*	*M. phlei*
P. nigrum	W	5	NZ	8
	E	NZ	12	12
	M	29	18	21
	C	10	6	NZ
C. zeylanicum	W	14	8	12
	E	7	NZ	NZ
	M	10	28	22
	C	NZ	NZ	12
E. cardamomum	W	NZ	NZ	6
	E	NZ	12	NZ
	M	19	25	14
	C	8	21	NZ
S. aromaticum	W	26	NZ	20
	E	NZ	24	NZ
	M	18	18	22
	C	10	NZ	NZ
Rifampicin		22	23	29

NZ=No Zone

Table 2 shows the MICs of the selected plant extracts with rifampin as a standard. The results showing that methanolic extract of *P. nigrum* and water extract of *S. aromaticum* shows MICs of 250 and 260 µg/ml respectively against *M.tuberculosis*. While methanolic extract of *E. cardamomum* and methanolic extract of *C. zeylanicum* having 250 µg/ml MICs against *M.smegmatis*. In case of *M. phlei*, methanolic extract of *C. zeylanicum*, *P. nigrum* and *S. aromaticum* shows same MICs 500 µg/ml.

The DPPH free radical scavenging activities of selected plant extracts are shown in the table 3. The methanolic and chloroform extract of *P. nigrum* Showed highly significant activity 92 and 90 % respectively also methanolic extract of *E. cardamomum* showed highest 94% while other extracts shows optimum DPPH radical scavenging activity, ascorbic acid were used as a reference for this assay.

Table 2: Minimum inhibitory concentration of the four extracts of the selected spices (µg/ml)

Name of spice	Extract	*M. tuberculosis*	*M. smegmatis*	*M. phlei*
P. nigrum	Water	400	NR	400
	Ethanol	NR	200	200
	Methanol	260	100	500
	Chloroform	400	400	NR
C. zeylanicum	Water	200	400	200
	Ethanol	400	NR	NR
	Methanol	400	250	500
	Chloroform	NR	NR	200
E. cardamomum	Water	NR	NR	400
	Ethanol	NR	200	NR
	Methanol	100	250	200
	Chloroform	400	360	NR
S. aromaticum	Water	250	NR	100
	Ethanol	NR	400	NR
	Methanol	100	360	500
	Chloroform	400	NR	NR
Rifampicin (µg/ml)		1.10	1.25	1.60

Results are the mean values of three independent experiments, NR=No reaction

Hydroxyl radicals are among the most hyper Reactive Oxygen Species and are considered to be responsible for some tissue damage occurring in inflammation. Results of hydroxyl radical (HO•) scavenging activity of selected plant extracts are shown in Table 3.

The results indicate that the methanolic extract of *P.nigrum* showed highest potential towards OH radical scavenging activity. Next to that water extract of *C. zeylanicum* and methanolic extract of *E. cardamomum* are having good activities, remaining extracts showed considerable potential for scavenge OH radical.

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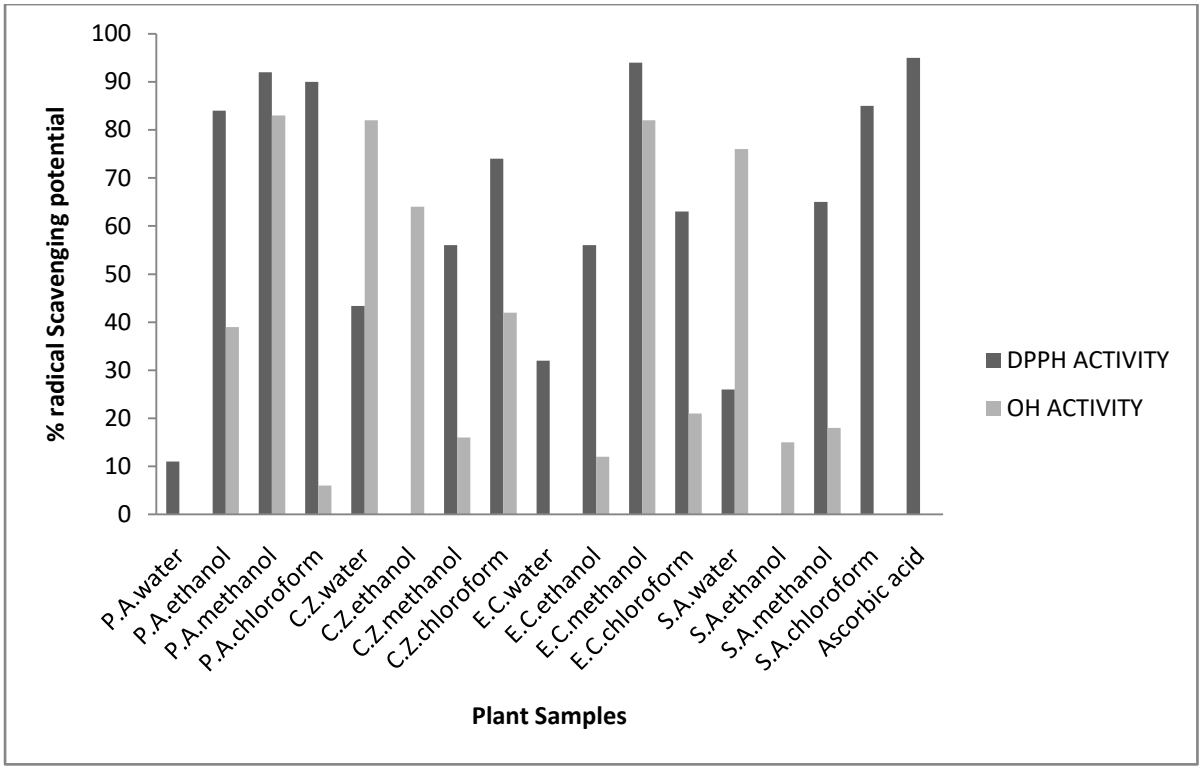

Figure 1: Antioxidant activities of the different extracts of selected spices

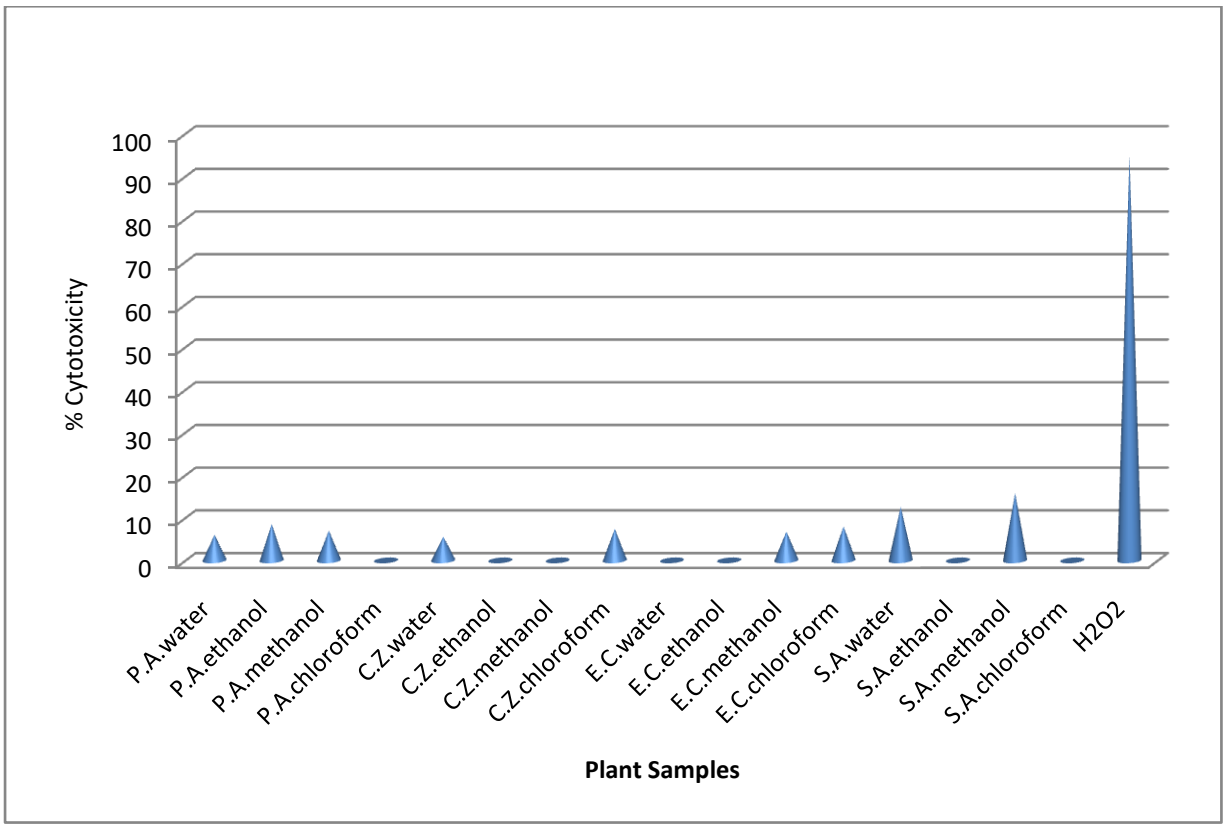

Figure 2: Cytotoxicity pattern of the different extracts of selected spices

The profile of the cytotoxicity is shown in Table 3. In metabolically active cells, MTT is reduced by the mitochondrial enzymes succinate dehydrogenase with the formation of insoluble purple formazan crystals. The maximum cytotoxic effect on chang liver cell lines is showed by the methanolic extract of the *S. aromaticum* upto 15% and all other extract showed negligible cytotoxicity.

Conclusion:

In the present investigation authors were tried to find the potential of some spices for their possible antimycobacterial inhibition. Along with this antioxidants and cytototoxicity studies are carried out in order to investigate their biological usefulness. These investigations will help the researchers over the globe, for the further investigation in the various treatments especially in the tuberculosis.

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OCULAR IN SITU GEL: A NOVEL APPROACH FOR BETTER PATIENT COMPLIANCE

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Abstract:

Ophthalmic *in situ* gelling systems (ISGs) at normal conditions behave like a liquid which undergoes phase transition in cul-de-sac under normal physiological condition to form stiff gel. Phase transition may occur due to alteration in pH, temperature or by interaction with ions and enzymes present in tear fluid. *In situ* formulations have proved its ability to sustained drug release with increasing the contact time of drug. Different polymers have proved their ability that to undergoes sol-to-gel phase transition which includes Poloxamers, Cellulose derivatives, Xyloglucan, Chitosan, Carbomer, Gellan gum, Alginic acid. Ophthalmic *in situ* formulation can be evaluated for various parameters like visual appearance, clarity, pH, gelling capacity, tonicity, *in vitro* drug release, rheology, texture analysis, *ex vivo* transcorneal permeability, ocular irritation studies by histological evaluation or HET-CAM test and accelerated stability testing.

Keywords: *in situ* gel; ophthalmic formulation; sustained release; transcorneal permeability study.

Introduction:

Eye is delicate sensory organ which converts light to electric signals that is treated and interpreted by brain (Kumar *et al.*, 2013). It gets suffer from various diseases like glaucoma, dry eye syndrome, trachoma, keratitis, conjunctivitis etc. (Rajoria and Gupta, 2012). Eye is unique organ in terms of its anatomical-physiological structure and defence mechanisms (Almeida *et al.*, 2013). It is highly protected organ which restrict the entry of any exogenous substances. Therefore, ophthalmic drug delivery system is foremost challenging endeavours facing by pharmaceutical scientist**.** Various problems associated with ocular drug delivery systems

includes nasolacrymal drainage of drug, binding of drug to lachrymal protein, induced lachrimation, availability of limited corneal area (Laddha and Mahajan, 2017).

For ailments of eye, administration of medicament through topical route is always preferable than the systemic administration. Drug can be delivered topically by solution, suspension ointments**.** Eye drops are most preferable dosage form as compared to other because of less cost and ease of administration but it possess poor contact time with eye and hence poor bioavailability **(**Patel *et al.,* 2013; Song *et al.,* 2013). It can be overcome by the use of *in situ* gel (ISG) system. The development of ISG systems has received considerable attention over the past few years. Recently, ISG forming system have showed their potential in increasing the residential time and possible controlled release of drug molecules for eye because of their capacity to improve bioadhesiveness of ophthalmic solution (Cao *et al.,* 2007; Laddha *et al.,* 2016). This system undergoes sol-to-gel phase transition in cul-de-sac which due to conformational changes in polymer in response to physiological parameters. Depending upon method employed to cause sol to gel phase transition on ocular surface three types of ISG are widely accepted as pH triggered systems, ion-activated systems and temperature sensitive system (Subimol *et al.,* 2013; Laddha and Mahajan, 2017).

Advantages of ocular *in situ* gelling systems

- Possibilities of administering accurate and reproducible quantities.
- Sustained drug release.
- Good ocular residence time.
- Reduces systemic absorption of drug.
- Use of biodegradable polymer.
- Ease of administration and reduces dosing frequency.
- Feasible for large scale production.
- Reduces cost of treatment.
- Improves patient compliance

Various approaches of ISG formation

Ideally, an *in situ* formulation should be free flowing liquid which allow ease of administration with reproducible dose delivery and upon instillation, undergoes to gel formation by phase transition should be strong enough to withstand the shear force in cul-de-sac which prolongs residence time of drug. It is widely accepted that increase in the viscosity of

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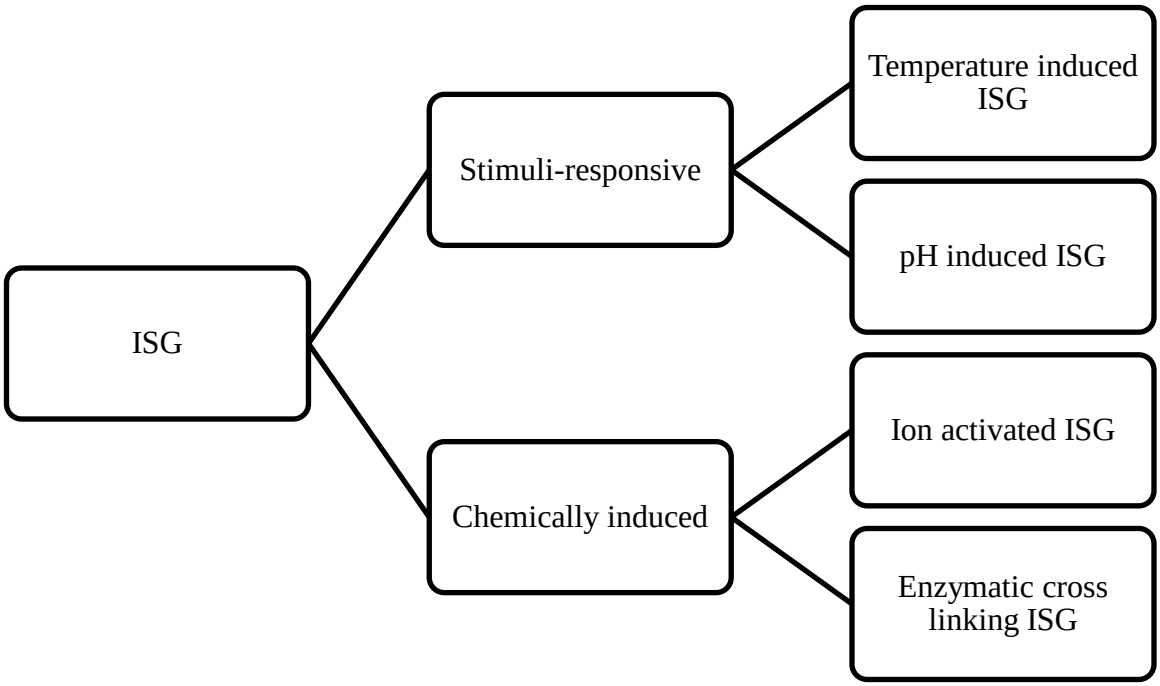
formulation in pre-corneal region leads to increase contact time and hence bioavailability. As *in situ* system undergoes phase transition in normal physiological condition and lead to form gel structure which results in increased bioavailability and less or no systemic side effect (Rajoria and Gupta, 2012).

Various approaches used for *in situ* gelling systems are as follows;



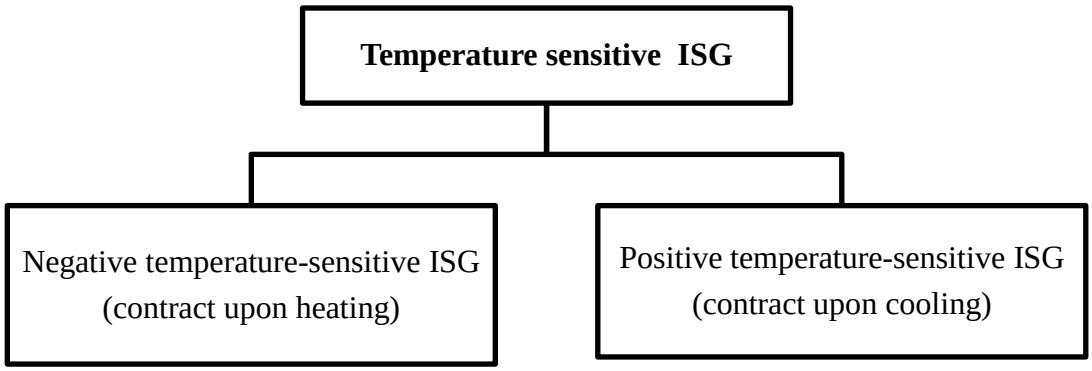
1. Stimuli responsive ISG

Stimuli responsive polymer also known as stimuli-sensitive, intelligent, smart or environmentally sensitive polymer undergoes physical or chemical changes in response to minute surrounding changes. These polymer systems may recognise a stimulus as a signal and then change their chain conformation in direct response (Kant *et al.*, 2011).

a) Temperature induced ISG system

Temperature-sensitive systems are the most commonly investigated type of ISG. As name itself indicates, this type of system utilises the polymer which undergoes sol-to-gel phase transition by change in temperature. Polymers design in a way that they undergo gel formation at physiological temperature.

Types of temperature sensitive ISG



Change in temperature leads to increase in degradation of polymer chains which leads to formation of hydrophobic domains and transition of an aqueous liquid to *in situ* gel (Kumar *et al.*, 2011; Laddha and Kshirsagar, 2021).

Examples of Polymer used in preparation of temperature induced ISG

Name of polymer	**Mechanism of gelation**
Poloxamers	Gradual desolvation of the polymer. Increased micellar aggregation. The increased entanglement of the polymeric network.
Cellulose derivatives	At elevated temperatures, these polymers adopt a random coil conformation
Xyloglucan	The lateral stacking of the rod like chains

b) pH induced ISGs

In this type of ISG change in pH is the factor contributing in phase transition. These types of polymers contain the acidic or basic functional group which either accepts or releases the proton due to change in pH. If polymer containing weak acidic group, then with increase in pH swelling of polymer increases and vice-versa.

Carbopol is well known pH sensitive polymer which is acidic by nature and undergoes phase transition above pH 4.2. This polymer and ISG is reported as safe, non-irritant and showed sustained release property (Laddha and Kshirsagar (2021); Laddha *et al.* 2016).

2. Chemically induced *in situ* gel system

a) Ion activated systems

Ion activated gelling system is triggered by cations present in eye tear fluid like Na^{+}, Ca^{+2} and Mg^{+2}. Generally anionic polymers are used in the formation of ion sensitive drug delivery system. Polymers like sodium alginate, gelrite, tamarind gum, gellen gum are used in combination with other polymers like MC and HPMC to increase the effect. They provide sustain release of drug by providing mucoadhesivenes.

Under this type of system ionic interaction between polymer and ions of tear fluid lead to form gel like structure. When anionic polymers come in contact with cationic ions they convert into gel. Person with normal physiology contains enough concentration of ions to cause this polymeric phase transition (Kant *et al.* (2011); Rajasekaran *et al.* (2010).

b) Enzymatic cross linking

In this approach, *in situ* formation is catalysed by natural enzymes present in tear fluid. However; limited investigations have been performed under this heading. It has the advantages over the other ISG like no need of any potential and harmful chemicals.

Evaluation of ISG:

Parameter	Need	Procedure
Visual appearance and clarity	Patient compatibility, Stability	Manual observation of formulation Visual appearance and clarity
pH	Patient comfort Drug solubility and stability	Measure by digital pH meter
Gelling capacity	To determine time require for sol-to-gel transition.	Place a drop of formulation in vial contains artificial tear fluid (2 ml) and determine time require for gel formation.
Tonicity	To prevent eye irritation	Measure by using osmometer
In vitro drug release study	To predict sustained release property of formulation	Perform by using Franz diffusion cell Place ATF in receptor compartment Place dialysis membrane is placed in between receptor and donor compartments.

		Place whole assembly is kept on the thermostatically controlled magnetic stirrer Maintain temperature at $37^{o}C \pm 0.5^{o}C$. Stir medium at 20 rpm. Place 1ml of formulation in donor compartment. Withdrawn 0.5 ml sample at predetermined time interval and same also replace equal volume with ATF. Samples analysis: UV spectrophotometer or HPLC
Viscosity	Determines flow property, residence time as well as patient compliance.	Determine viscosity before and after gelation by using Brookfield viscometer preferably with small volume adapter.
Texture analysis	To determine the consistency, firmness, and cohesiveness	Determine by using texture analyser.
Transcorneal permeability study	To determine corneal permeability of by *ex vivo*	By using goat eye cornea. Procedure: same as *in vitro* drug release study. Use isolated cornea in place of dialysis membrane and perform experiment for 5 h.
Ocular irritation potential	To irritation capacity of formulation	Histology study of goat eye cornea obtained after transcorneal permeability study.
Accelerated stability study	To determine stability of formulation.	Perform as per ICH guidelines. Place formulation at elevated temperatures and humidity conditions of 25±1°C/ 60%RH, 30±1°C/ 65%RH and $40 \pm 2^{o}C$/ 75 ± 5 % RH. Withdraw ample at the end of 0, 30, 60 and 90 days and then evaluate for active drug content

Conclusion:

Poor ocular bioavailability and therapeutic response shown by conventional ophthalmic system can be overcome by use of ISG system which undergoes reversible sol to gel transition in cul-de-sac by physical stimulation. It is an ideal system which increases patient compliance by prolonging ocular residence time of formulation which reduces dose, dosing frequency and hence cost of treatment. This system is preferred over other systems for ocular delivery because it can be administered in drop form which deliver accurate dose with fewer problems with vision. Further use of biodegradable and water soluble polymer makes this system more acceptable.

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DESIGN AND EVALUATION OF PUSH PULL OSMOTIC PUMP TABLETS BY COATING THE INDENTED CORE TABLETS

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Abstract:

The purpose of this work was to develop extended release osmotic tablets of highly water soluble metoprolol tartrate (MT) using PPOP (Push Pull Osmotic Pump) system. This is cost effective, elimination of laser drilling and avoidance of complicated side identification during laser drilling. Formulation consists of two layers; at varying concentration of drug ratio in upper layer and lower layer, along with polymeric osmotic agent like HPMC (Hydroxy Propyl Methyl Cellulore/ SCMC (Sodium Carboxy Methyl Cellulore). The bilayer core tablets are compressed and an indentation at diameter of 1.0, 0.8, 0.6 mm are produced at the center of drug layer surface. The PPOP tablet release showed zero order release kinetics. However, selection of best formulation according to the similarity factor (f2). HA3 was found to be the most similar formulation to marketed product (reference standard). The Scanning Election Microscopy(SEM) studies were conducted to elucidate the changes in the membrane structure and orifice diameter before coating, after coating and after in-vitro dissolution studies. Detecting any possible interaction between drug and polymers in the HA3 formulation was seen as confirmed by FTIR and DSC studies. In above formulations, HA3 formulation showed precise extended release of MT by PPOP system.

Keywords: Metoprolol tartrate Push Pull Osmotic Pump; Osmotic drug delivery system; Controlled release; Multidrug therapy

Introduction:

A number of design options are available to modulate the drug release from a dosage form and majority of them fall in the category of Matrix, reservoir (or) osmotic system. Among various controlled release devices, osmotically driven system hold a prominent place because of their reliability and ability to deliver the contents at predetermined rate for prolonged periods.[1,3,4] The osmotic pump is similar to a reservoir device but contains an osmotic agent (eg:- the active

agent in salt form), which imbibes water from the surrounding medium via semipermiable membrane. Such a device called the EOP (Elementary Osmotic Pump) has been described by theeuwes. . Normally EOP delivers 60-80% of its content at a constant rate and there is short lag time of 30-60 min[2,13]. As the system hydrates zero order delivery from the EOP is obtained. EOP is limited to the delivery of relatively soluble drugs (50-300mg/ml) [2]. The push pull osmotic pump tablets are developed in the 1980s. PPOP is complementary in design to the EOP. However the PPOP is desirable, for the delivery of poorly water soluble. (Or) very water soluble drugs at constant rate. DitropanXL (Oxybutynin chloride), ProcardiaXL (nifedipine) and glucotrol XL (glipizide) are core molecule fabricated as PPOP based on this technology. Push pull pumps delivered about 80% of drugs at zero order rate.[3, 11, 12.]

Compared with the monolithic osmotic pump tablet (OPT), the bilayer-core OPT has several advantages, [11,12,13,14]

- It was more suitable for delivering very water soluble drugs.
- Its release rate is much closer to zero order rate.(Higher fractions of drug at zero order')
- A higher cumulative released percentage at 24 hrs could be achieved.

PPOP is the development of modified two layer osmotic pump tablet whose core tablet consist of two layers, one layer containing drug and the other containing an osmotic agent and an expanding agent.

Disadvantages of PPOP system[15]

- Complicated side identification technology should be employed to ensure the orifice drilled on the surface of the drug layer after coating.
- An expensive laser drilling machine was necessary for the large scale preparation of all kinds of osmotic pump tablets.

In this work, the above mentioned problems were eliminated by compressing the two layered modified osmotic pump tablet using modified punches.[14, 15, 18.]So, this work is simplified with the elimination of 'LASER DRILLING'. Thus the core tablet with an indentation is prepared by compression by the modified punch with a needle and then the two layered osmotic pump tablet is achieved by coating the indented tablet. Osmotic control drug delivery system has found to produce more significant use in pharmacotherapy of cardiovascular diseases.[19, 20, 21.]Metoprolol tartrate is selected as model drug in the preparation of osmotic pump tablet. It is a cardio selective adrenergic blocking agent and widely used as a drug of choice in the management of hypertension, angina pectoris and arrhythmias. The drug is freely soluble in

water and administered at a dose of 100mg daily, the half life of metoprolol tartrate is about 3-4 h and oral bioavailability has been reported to be about 50%.

Since, the half life of Metoprolol tartate is 3-4 hour multiple doses are needed to maintain constant plasma concentration for a good therapeutic response and improved patient compliance. Hence, an attempt is made to formulate a bi layered osmotic tablet to regulate the release of Metoprolol tartate by using PPOP system to extend the clinical effect, reduce dosing frequency, avoid dose dumping, maintain uniform plasma concentration of drug and increase the patient compliance.

Experimental:

Materials

Metoprolol tartrate was obtained as a gift sample from paris dackner pvt.Ltd., HPMC K100M/K4M gift sample from Micro Labs., SCMC gift sample from CDH Lab., Cellulose Acetate gift sample from LOBA chemie private limited.

Methods:

DSC[6,33,40]**/ FTIR**[40,41]

Chemical interaction between drug and polymers in the HA3 formulation was seen as confirmed by FTIR and DSC studies. The possibility of drug-excipient interaction was investigated.

Preparation of modified two layered indented PPOP tablets:[7,8,9,10,14,16,17,19,20..]

Preparation of pull and push layer granules:

Six formulations of metoprolol tartrate are prepared with two Layers. Each layer contains varying concentration of drug. (100:0; 50:50: 25:75).Granules of push and pull layer were prepared by wet granulation method. Push and Pull layer ingredients, filers and along with the polymers and osmogen was mixed separately using acetone as solvent, The acetone was added to drug layer ingredients and separately to push layer form a damp mass and passed through sieve No.10, and dried at room temperature to cease acetone followed by drying at hot air oven at 40^0 c for 30 min; Dried granules was passed separately through a sieve number #25 and #22 to get uniform particle size. Prepared granules then lubricated with talc and magnesium sterate, and mixed well.Push layer (lower layer) contains high molecular weight polymer (HPMC

K100M)/SCMC (High viscosity) and upper layer contains low molecular weight polymer (HPMC K4M) / SCMC (Low Viscosity). The composition shown in Table 1.

Table 1: Composition of core tables

Ingredients	**SA**	**SB**	**SC**	**HA**	**HB**	**HC**
Drug Layer						
Metoprolol tartrate	50	25	12.5	50	25	12.5
Sodium CMC (low viscosity)	25	25	25	-	-	-
HPMC-K 4M	-	-	-	25	25	25
Pvp k-30	12	12	12	12	12	12
Lactose	79	104	116.5	79	104	116.5
Sodium chloride	50	50	50	50	50	50
Talc	12	12	12	12	12	12
Megnisium state	12	12	12	12	12	12
IPA	qs	qs	qs	qs	qs	qs
Push Layer						
Metoprolol tartrate	-	25	37.5	-	25	37.5
Sodium CMC (High viscosity)	25	25	25	-	-	-
HPMC-K 100M	-	-	-	25	25	25
Pvp k-30	8	8	8	8	8	8
Lactose	86	61	48.5	86	61	48.5
Sodium chloride	25	25	25	25	25	25
Talc	8	8	8	8	8	8
Megnisium sterate	8	8	8	8	8	8
Erythrosine	qs	qs	qs	qs	qs	qs
IPA	qs	qs	qs	qs	qs	qs

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The resultant granules are compressed into core tablet using a Single Punch Tableting Machine-(cadmach), whose upper concave faced punch was modified with a needle of different diameter[1,14,15,18]. The push layer granules are placed first into the die cavity, and subsequently the drug layer granules are loaded. Finally, the bilayer core tablets are compressed and an indentation at diameter of **1.0mm, 0.8mm, 0.6mm** and depth of **1.50mm** are produced at the center of drug layer surface. The weight of each tablet is maintained within the range of 380 to 420 mg. Hardness of the core tablets were with 5-7kg/cm$^{2.}$ Osmotic tablets which different delivery orifices. Total 18 formulation code was tabulated in table 2.

The drilled tablets are coated according to their orifice diameters and were used for further studies.

Table 2: Osmotic tablets with different delivery orifices

CODE		
S	**Sodium carboxy methyl cellulose.**	
H	**Hydroxy propyl Methyl cellulose.**	
A	**100% drug in drug layer.**	
B	**50% drug in drug layer.**	
	50% drug in push layer.	
C	**25% drug in drug layer.**	
	75% drug in drug layer.	
1	**1.0mm.**	**Depth 1.5 mm**
2.	**0.8mm.**	
3.	**0.6mm**	

Eighteen Formulations are coded

HA- HPMC -100% Drug in Drug layer.
HB- HPMC -50% Drug in Drug Layer.
-50% Drug in Push Layer.
HC- HPMC -25% Drug in Drug Layer.
-75% Drug in Push Layer.
SA- SCMC -100% Drug in Drug layer.
SB- SCMC -50% Drug in Drug Layer.
-50% Drug in Push Layer.
SC- SCMC -25% Drug in Drug Layer.
-75% Drug in Push Layer.

HA-**1**-(1.0mm), HB -**1**-(1.0mm), HC-**1**-(1.0mm).
HA-**2**-(0.8mm), HB -**2**-(0.8mm), HC-**2**-(0.8mm).
HA-**3**-(0.6mm), HB -**3**-(0.6mm), HC-**3**-(0.6mm).
SA- **1**-(1.0mm), SB -**1**-(1.0mm), SC- **1** -(1.0mm).
SA- **2**-(0.8mm), SB -**2**-(0.8mm), SC- **2** -(0.8mm).
SA- **3**-(0.6mm), SB -**3**-(0.6mm), SC- **3** -(0.6mm).

Total 18 Formulation code.

Coating of core tablets:

Preparation of coating solution: [30.]

5 % w/w solution of Cellulose acetate (CA) in Dichloromethane and Methanol mixture (4:1) containing PEG 400 as plasticizer in the concentration of 15%w/w (w.r.t. cellulose acetate) is used as an optimized formula to coat the tablets, as it shown to exhibit required permeability characteristics and remain rigid after 12 hours.

Coating procedure: [20, 24, 30.]

Coating is done by using Pan coating machine. The coated tablets are evaluated for membrane uniformity, thickness and orifice diameter by Scanning Electron Microscopy (SEM).

Evaluation of Granules/Uncoated and coated Tablets[22.]

The powder blends of all the formulations were evaluated for bulk density, tapped bulk density, angle of repose and carr's index in flow/ compressibility index.

The uncoated tablets were biconvex, bilayered circular shape tablet with pink on push layer and white on drug layer with indentation. The tablets were tested for hardness, thickness, friability, weight variation, percentage of drug content[23]

The coated tablets were half white in colour, circular and biconvex in shape. They were glossy and elegant in appearance. Parameters like percentage of weight increase[20, 22.] Of coated tablet, drug content analysis[23] were presented in table 4.

In vitro dissolution studies: [2, 16,19,25,26,32.]

In vitro release study was performed in a USP Dissolution Apparatus Type II using the paddle method. The dissolution media used is Hydrochloric acid buffer pH 1.2 (900 ml) for first 2 hrs and Phosphate buffer pH 7.4 (900 ml) for subsequent 10 hrs. The temperature and the stirring rate are maintained at 37±1° C and 50±1 rpm respectively. 5 ml of samples were withdrawn at intervals of 1 hr and the same volume of corresponding dissolution medium was replenished to maintain a consistent volume. The amount of metoprolol tartrate released is determined by measuring the absorbance of the samples at 222 nm using UV-Visible Spectrophotometer. Each test is performed in triplicate.

Drug release kinetics [20,26,35,36,37,38,39.]

To study the release kinetics, data obtained from in vitro drug release studies were plotted in various kinetic models zero order as cumulative amount of drug released vs time(eqn.1), first order (eqn.2)as log cumulative percentage of drug remaining vs time, Higuchi's model (eqn.3) as cumulative percentage of drug released vs root of time and Hixson-crowell

cube root law to evaluate the drug release with changes in the surface area and the diameter of the particles/tablets.

The data were evaluated according to the following equations:

- Zero order - $Q = K_0 t$
- First order - $\text{Log } Q = \text{Log } Q_0 - kt /2.303$
- Higuchi - $Q_t = k_H t^{1/2}$
- Hixson-crowell - $Q_0^{1/3} - Q_t^{1/3} = k_{HC}t$.

To evaluate the mechanism of drug release was plotted in korsmeyer – peppas. Equation, $Mt / M00 = Kt^n$. Log time vs cumulative % drug release. **n-**is an exponent that characterizes the mechanism of release traces.

Similarity factor (f2 value)[14,15,29.]

The similarities between two dissolution profiles of formulation and reference standard were assessed by a pair wise model independent procedure such as similarity factor (f2).

Scanning Electron Microscopy (SEM):-[5,20,29,34,38.]

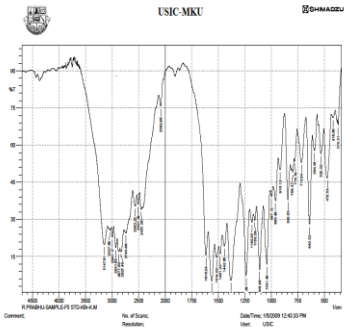
The SEM studies were conducted to elucidate the changes in the membrane structure and orifice diameter before coating, after coating and after in-vitro dissolution studies (JFC-1100,Jeol,Japan)

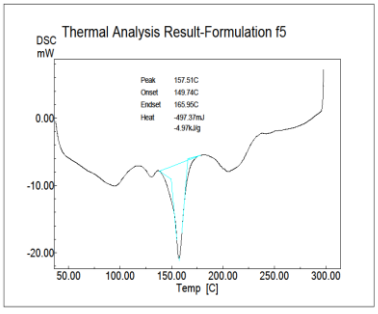
Results and Discussion:

DSC[40, 42]**/ FTIR**[41,44]

Chemical interaction between drug and polymers in the HA3 formulation was seen as confirmed by FTIR and DSC studies. The possibility of drug-excipient interaction was investigated. It shows that there was no specific interaction between the drug and the excipients. In DSC curves, thermograms can be used to check the purity of the compound.DSC thermograms of the pure drug and the HA3 formulation. From the thermogram it was clear there was no specific interaction between the drug and excipients used in the formulations.

F-5: Formulation: FT-IR **DSC**





Evaluation of Granules/Uncoated and coated Tablets[22,23,27,28,24]

The values of Bulk density, Tapped bulk density, Carr's index and Angle of repose are showed good physical properties of granules. Flow property and compressibility ensured uniform distribution of drug in granules. All the values were found to be within the IP limits.

The uncoated tablets were biconvex, bilayered circular shape tablet with pink on push layer and white on drug layer with indentation at diameters 1.0mm,0.8mm,0.6mm and depth 1.50mmwere produced at the centre of drug layer surface.[1,11,12,15] Weight of each tablet was maintained within the range of 380-420 mg.

The coated tablets were off white in colour, circular and biconvex in shape. They were glossy and elegant in appearance. Parameters percentage of weight increase[20,22.],thickness of coated tablet increases 0.6 mm and drug content analysis found to be within the IP limits (± 10%)[29]. All the values hardness, friability, weight variation given in table 4. All the values were found to be within the IP limits. And results showed in table-3.

Table 3:

Ingredients	**SA**	**SB**	**SC**	**HA**	**HB**	**HC**
Drug Layer						
Bulk Density(gm/ml)	0.51	0.45	0.56	0.56	0.54	0.54
True Density(gm/ml)	0.66	0.62	0.70	0.71	0.67	0.70
Compressibility Index %	22.0	26.4	21.0	21.0	19.0	22.0
Drug Content %	98.85	100.37	101.1	100.0	100.0	98.88
Angle of Repose ϕ	26.59	31.21	27.42	31.5	31.5	28.2
Push Layer						
Bulk Density(gm/ml)	0.49	0.46	0.46	0.46	0.50	0.47
True Density(gm/ml)	0.68	0.64	0.64	0.68	0.63	0.71
Compressibility Index %	27.6	28.0	28.0	32.0	22.0	34.0
Drug Content %	-	99.62	98.5	-	99.25	100.0
Angle of Repose	25.18	26.18	25.78	26.37	26.18	30.3

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Table 4: (a) SCMC

Evalution Parameters	SA1		SA2		SA3	
	Uncoated	Coated	Uncoated	Coated	Uncoated	Coated
Hardness (kg/cm^2)	7.6	11.0	6.6	11.0	5.1	11.0
MembranceThickness (mm)	4.2	4.8	4.1	4.7	4.2	4.8
Weight variation %	403.1	442.1	398.3	436.3	407.2	449.3
Fribility of tablets %	0.54	0.003	0.67	0.01	0.36	0.02
Drug content %	96.65	97.89	95.91	98.14	97.76	99.62

SA1- SCMC 1.0MM SA2- SCMC 0.8MM SA3- SCMC 0.6MM

Allvalues are mean ± S.D for n=3

(b) HPMC

Evalution Parameters	HA1		HA2		HA3	
	Uncoated	Coated	Uncoated	Coated	Uncoated	Coated
Hardness (kg/cm^2)	7.3	11.0	6.6	11.0	5.5	11.0
MembranceThickness (mm)	4.3	4.9	4.2	4.8	4.2	4.8
Weight variation %	405.0	444.0	397.1	434.7	398.6	441.1
Fribility of tablets %	0.44	0.01	0.56	0.01	0.66	0.01
Drug content %	99.25	100.37	98.88	98.14	101.85	98.51

HA1- HPMC 1.0MM HA2- HPMC 0.8MM HA3- HPMC 0.6MM

All values are mean ± S.D for n=3

***In vitro* dissolution studies:**

The formulations were subjected to in-vitro dissolution studies for 12 hrs. The dissolution media was acid buffer pH 1.2 for first 2 hrs and Phosphate buffer pH 7.4 for the next 10 hrs.

Effect of polymers on drug release:- [17,19,26]

The present study was aimed to control the drug release from osmotic pumps by the use of hydrophilic polymers which act as retardants and they form hydrogel inside the device on exposure to the external aqueous environment.

The cumulative percentage drug release from formulations with **SCMC** at the end of 10 hrs was found to be

SA1 -99.9% ; SB1-99.6% ; SC1-83.9%.....1.0mm (Fig-1)

SA2 -101.9% ; SB2-103.6% ; SC2-97.1%.....0.8mm (Fig-2)

SA3-100.0% ; SB3-100.7% ; SC3-93.5% .....0.6mm (Fig-3).

For marketed product 64.5%.

The cumulative percentage drug release from formulations with **HPMC** at the end of 12 hrs was found to be

HA1-64.5% ; HB1-57.1% ; HC1-60.3%.....1.0mm (Fig-4)

HA2-68.6% ; HB2-58.0% ; HC2-55.5%.....0.8mm (Fig-5)

HA3-70.3% ; HB3-64.2% ; HC3-39.4%.....0.6mm (Fig-6)

For marketed product 64.5%.

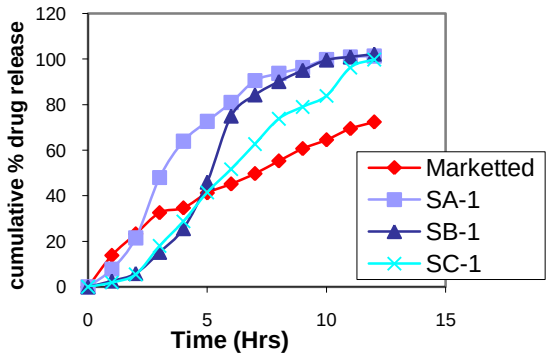

Figure 1: *In vitro* dissolution studies of PPOP – S- 1.0 mm

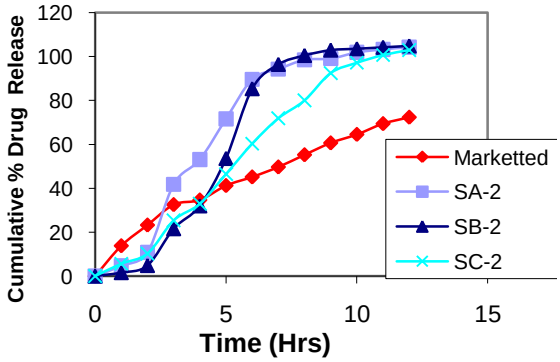

Figure 2: *In vitro* dissolution studies of PPOP - S- 0.8mm

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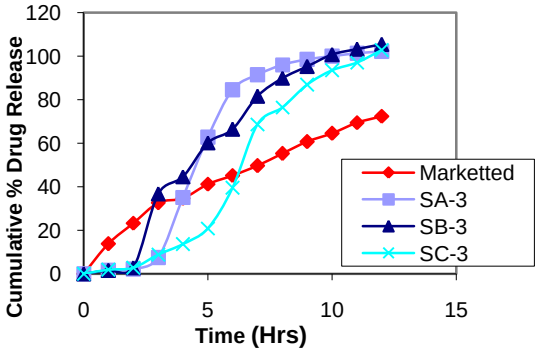


Figure 3: *In vitro* dissolution studies of PPOP – S- 0.6 mm

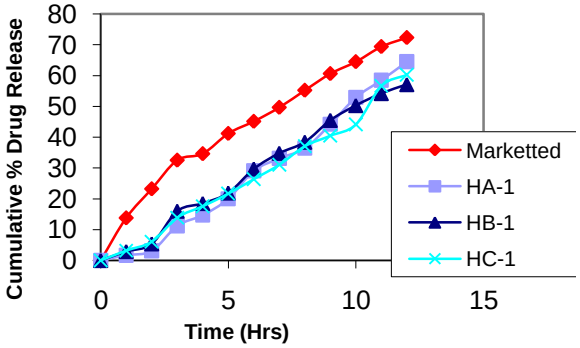


Figure 4: *In vitro* dissolution studies of PPOP – H- 1.0 mm

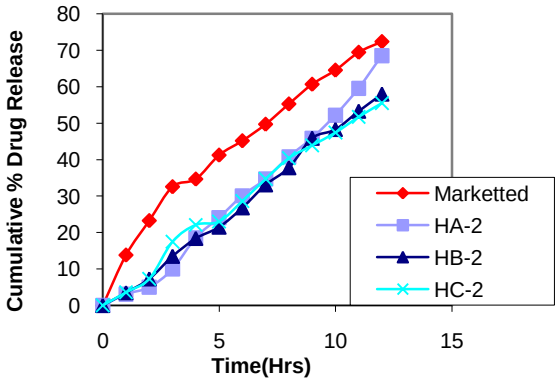


Figure 5: *In vitro* dissolution studies of PPOP – H- 0.8 mm

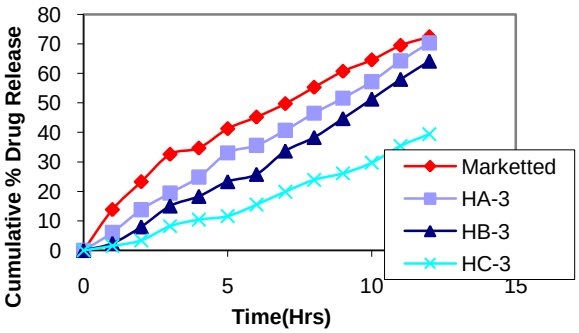


Figure 6: *In vitro* dissolution studies of PPOP – H- 0.6 mm

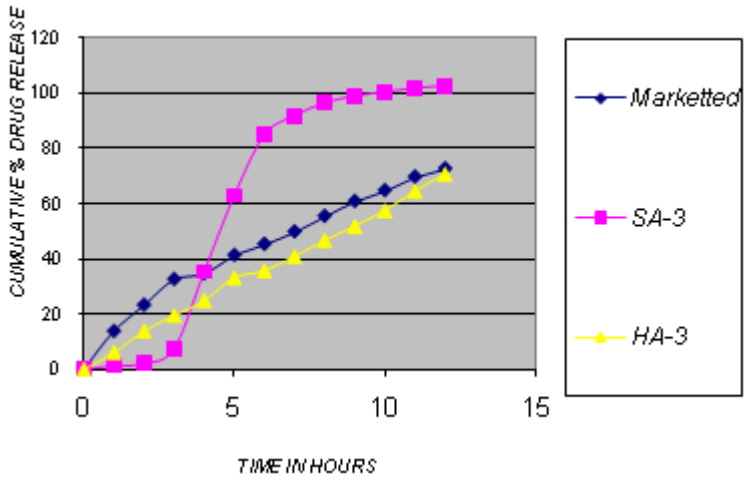


Figure 7: shows the comparative drug release profile of formulations containing constant amounts of SCMC, HPMC (0.6mm indentation) and Marketed formulation

The results showed that the osmotic devices containing SCMC disintegrated after a few hours of exposure to the dissolution medium. This effect may be due to high swelling power of SCMC. In other words, disintegration occurred probably as a result of greater rate of polymer swelling (volume expansion) than the rate of the swelled polymer departure through the orifice; this could increase the pressure within the device resulting in disintegration of the device after a few hours of exposure to dissolution medium[21].

Furthermore, these polymers also can produce a highly viscous solution after the exposure to dissolution medium which may block the orifice of the device and consequently increase the internal pressure of the system and possible rupture of the semipermiable membrane coating. Due to this effect, in formulations containing SCMC, the release rate was very high. At the same time osmotic devices containing HPMC remained intact after 12 hours exposure to the dissolution medium. These formulations also showed retarded release of drug from 0-12 hrs. When compared to formulation containing SCMC.

These results demonstrated that HPMC may be suitable for metoprolol PPOP system to obtain zero order release and reasonable amount of drug release for a period of 12 hours.

Effect of concentration of drug in two layers:-[20.]

The effect of concentration of drug on drug release was studied by preparing osmotic tablets containing different proportion of drug in drug layer and push layer (100:0; 50:50 ; 25:75).The designing of the system involved keeping metoprolol tartrate into two different layers in varying amounts and different invitro release profiles of metoprolol tartrate obtained.

Formulations containing 100% drug in upper layer (drug layer HA1/HA2/HA3) showed higher release rate when compared to formulations containing drug in both layers (HB1/HB2/HB3 – HC1/HC2/HC3)

The results indicated that formulation HA3 showed approximately similar release profile of marketed product and release from other formulations significantly varied from the marketed product.

Similarity Factor (f2) Value:

The similarity factor (f2) also confirms the same.The results indicated that formulation HA3 showed approximately similar release profile of marketed product. The factor f2 measures the closeness between the two profiles.HA3 and marketed product.

Effect of Orifice diameter [14,15,16,17]

- From the results it was clear that no significant difference in the release profile from osmotic device of orifice diameter ranging from 0.6 to 1.0 mm.
- Drug release from osmotic system is not affected by the size of the delivery orifice within the certain limits [0.25 to 1.41mm].[1.]

Mechanism and kinetics of drug release[35,36,37,38,39,43.]

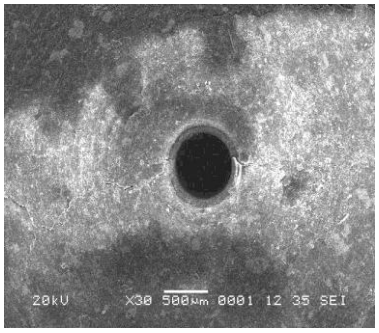
The in-vitro Metoprolol tartrate release data were fitted to Korsmeyer-Peppas release model and exponent values (n) were calculated.

For formulation HA3, the release exponent value observed was 0.9471, indicating the release mechanism of metoprolol tartrate from the osmotic pump tablet follows super case II transport, where drug release is due to polymer dissolution. This formulation also showed higher R^2 values for "zero order" kinetics indicating the metoprolol tartrate release from this osmotic pump tablets were by both diffusion and erosion.

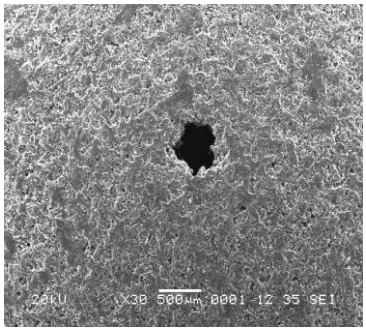
From the results it was observed that formulation HA3 containing HPMC and 100% drug in upper layer may be the suitable formulation which showed zero order release kinetics and reasonable amount of drug release for a period of 12 hrs release kinetics data are shown in Table 5.

Scanning Electron Microscopy (SEM):-[5,31,33]

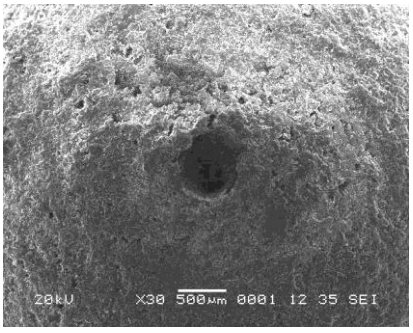
The SEM studies were found that there were no significant changes in the membrane structure and orifice diameter before coating, after coating and after in-vitro dissolution studies.



Before Coating



After Coating



After Dissolution

Table 5: Release kinetics

Code	f2	Zero order		First order		Higuchi		Hixson crowell		Korsmeyer Peppas		
		R2	$K_0(h^1)$	R2	K_H(h1)	R2	K_H(h-1/2	R2	$K_{HC}(h^{1/3})$	R2	Kkp(h-n)	n
SA1	26.0	0.8512	8.18	0.8421	0.513	0.938	39.67	0.9484	0.505	0.8533	8.43	0.989
SA2	24.6	0.8441	9.31	0.9284	0.518	0.923	45.00	0.9600	0.618	0.7919	4.35	1.270
SA3	25.7	0.8554	10.7	0.9502	0.523	0.903	50.95	0.9661	0.587	0.6940	0.76	1.976
SB1	29.0	0.9154	10.6	0.8830	0.517	0.937	49.52	0.9235	0.553	0.8374	1.97	1.621
SB2	25.0	0.873	10.8	0.8882	0.545	0.918	51.49	0.9198	0.724	0.7495	1.33	1.771
SB3	28.8	0.9434	9.99	0.9254	0.481	0.971	47.07	0.8949	0.607	0.8169	1.27	1.763
SC1	38.0	0.9884	9.49	0.6562	0.437	0.981	43.69	0.9032	0.333	0.9437	1.80	1.621
SC2	33.0	0.9755	9.77	0.8899	0.445	0.978	45.20	0.8576	0.514	0.9572	4.93	1.250
SC3	32.0	0.9513	10.8	0.8674	0.399	0.912	49.22	0.8137	0.449	0.9155	1.15	1.853
HA1	38.9	0.9922	5.88	0.9593	0.091	0.951	26.58	0.9746	0.121	0.9898	1.48	1.520
HA2	41.3	0.9939	5.94	0.9515	0.095	0.956	26.89	0.9720	0.125	0.9949	2.59	1.307
HA3	**52.9**	**0.9968**	**5.68**	**0.9697**	**-0.04**	**0.978**	**25.65**	**0.9851**	**0.124**	**0.9968**	**6.49**	**0.947**
HB1	40.1	0.9932	5.65	0.9894	-0.03	0.977	23.47	0.9931	0.103	0.9847	2.75	1.228
HB2	39.2	0.9966	5.07	0.9833	0.076	0.969	23.08	0.9903	0.102	0.9959	3.46	1.139
HB3	40.7	0.9932	5.48	0.9579	-0.03	0.955	24.82	0.9739	0.114	0.9961	2.85	1.242
HC1	38.1	1.9876	5.10	0.9497	0.077	0.950	23.13	0.9663	0.103	0.9905	3.15	1.178
HC2	40.1	0.9887	4.71	0.9925	0.070	0.984	21.70	0.9939	0.094	0.9849	3.64	1.092
HC3	27.0	0.9889	3.40	0.9734	0.043	0.943	15.37	0.9796	0.061	0.9952	1.53	1.299
MP*	-	0.9864	5.10	0.9906	0.100	0.993	23.65	0.9952	0.123	0.9960	14.3	0.648

Summary and Conclusion:

The present work aimed towards developing modified push pull osmotic pumps of metoprolol tartrate. Among various controlled release devices, osmotically driven system holds a prominent place because of its reliability and ability to deliver the contents at predetermined rate

- This work is simplified with the elimination of 'LASER DRILLING'. Thus the core tablet with an indentation is prepared by compression by the modified punch with a needle and then the two layered osmotic pump tablet is achieved by coating the indented tablet. The indented osmotic pump tablet were prepared with two layers.
  - Each layer contains varying concentration of drug.(100:0 ; 50:50 : 25:75).
  - Push layer contains high molecular weight polymer (HPMC K100M)and upper layer contains low molecular weight polymer (HPMC K4M).
  - All bilayer core tablets indented with different orifice diameters (0.6 ; 0.8 ; 1.0 mm).
- The formulated tablets were having enough hardness and friability and they are within the IP limits. This shows that the tablets are capable of withstanding mechanical shock during handling, packing and shipping.
- The percentage weight increase after coating was found to be uniform in all the formulations. The coating remained intact even after 12 hours. The orifice diameters were measured using SEM.
- All the formulations showed uniformity in weight and drug content was within the IP limits. These reveal that the drug is uniformly distributed in the tablets.
- The in-vitro drug release studies showed that the cumulative percentage drug release was controlled by the presence of SCMC and HPMC. From the results, it was found that the retarding capacity of the polymers is in the following order:**HPMC > SCMC**

From the above points it is evident that HPMC is the first choice to obtain zero order release and expected amount of drug release for a period of 24 hours.

- Indentation size (0.6; 0.8; 1.0 mm) of core tablet hardly affected the drug release from the formulations.
- The best formulation was selected based on similarity factor by comparing the in-vitro release profile with the marketed drug release profile.
- Drug release Kinetics suggests that the higher correlation coefficient (0.9968) values the release data seen to better fit with zero order release.
- DSC/FTIR studies the possibility of drug-excipient interaction was investigated. It shows that there was no specific interaction between the drug and the excipients.
- The results showed that the PPOP can be a very effective device for the delivery of water soluble drug with zero order release pattern.
- This method may be promising in the field of the preparation of osmotic pump tablet.

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A PRACTICAL APPLICATIONS AND EXPERIMENTAL STUDIES ON PROTOPLAST ISOLATION

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Abstract:

The protoplast, also known as naked plant cell refers to all the components of a plant cell excluding the cell wall. Hanstein introduced the term protoplast in 1880 to designate the living matter enclosed by plant cell membrane (Venkateshwarlu, 2020). The isolated protoplast is unusual because the outer plasma membrane is fully exposed and is the only barrier between the external environment and the interior of living cell. The isolation of protoplasts from plant cells was first achieved by microsurgery on plasmolyzed cells by mechanical method. However, the yields were extremely low and this method is not useful (Venkateshwarlu, 2018; Latha and Venkateshwrlu, 2019). Researcher isolated the enzyme cellulase from the culture of fungus *Myrothecium verruca/in* to degrade the cell walls. He applied an extract of cellulase hydrolytic enzyme to isolate protoplasts from tomato root tips. Since that time many enzyme formulations have been used to isolate protoplasts, and the most frequently used enzymes are now commercially available.

Keywords: Protoplast Isolation, Practical Applications, Experimental Methods.

Introduction:

The use of cell wall degrading enzymes was soon recognized as the preferred method to release large numbers of uniform protoplasts. Under appropriate conditions, in a number of plant species, these protoplasts have been successfully cultured to synthesize cell walls and now genetic manipulations have also been made. Protoplasts are not only useful for ceil fusion studies but, these can also take up, through their naked plasma membrane foreign DNA, cell organelles, bacteria or virus particles (Vasil *et al.*, 1980). Regeneration of a cell wall is not necessarily a prerequisite for the initiation of nuclear division in protoplast cultures but cell wall formation is required before cytokinesis occurs. Once a cell wall is formed, the reconstituted cells show a considerable increase in cell size. The first cell division generally occurs within 2-7 days (Takebe *et al.*, 1971). Protoplasts from actively dividing cell suspension enter the first division faster than those from highly differentiated cells of the leaf (Cocking, 1960; Bajaj, 1989; Ugender *et al.*, 2017). The second division occurs within a week, and by the end of the second

week in culture, small aggregates of cells are present. After 3 weeks, small cell colonies are visible and colonies of *ca.* 1 mm diameter are present after approximately 6 weeks in culture. Once small colonies have formed, their further growth is inhibited if they are allowed to remain on the original high osmotic medium. The colonies should, therefore, be transferred to a mannitol or sorbitol-free medium.

Protoplast isolation:

Protoplasts are isolated by (i) mechanical and (ii) enzymatic methods.

1. **Mechanical method**

In this method, large and highly vacuolated cells of storage tissues such as onion bulb scales, radish root and beet root tissue could be used for isolation. The cells are plasmolysed in an iso-osmotic solution resulting in the withdrawal of contents in the center of cell. Subsequently, the tissue is dissected and deplasmolyzed to release the preformed protoplasts T. Nagata (1971). Isolated protoplasts from *Stratiotes abides* by this method. Mechanically, protoplasts have been isolated by gently teasing the new callus tissue initiated from expanded leaves of *Saintpaulia ionantha* which was grown in a specific auxin environment to produce thin cell walls. However, this method is generally not followed because of certain disadvantages:

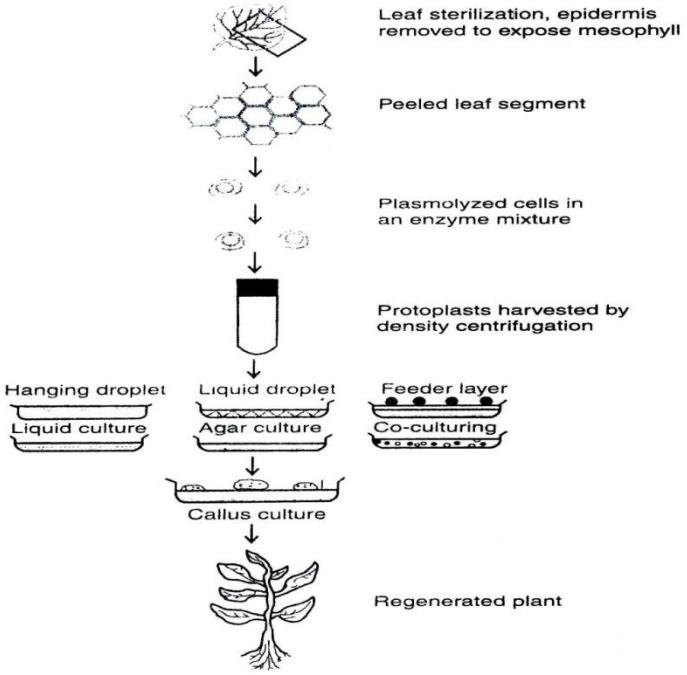

Figure 1: Schematic illustration of protoplast isolation and culture procedure

(i) It is restricted to certain tissues which have large vacuolated cells;

(ii) Yield of protoplasts is generally very low. Protoplasts from less vacuolated and highly meristematic cells do not show good yield,

(iii) The method is tedious and laborious;

(iv) Viability of protoplasts is low because of the presence of substances released by damaged cells. The mechanical method is useful when there are side effects of cell wall degrading enzymes.

2. Enzymatic method

Cocking in 1960 demonstrated the possibility of enzymatic isolation of protoplasts from higher plants. He used concentrated solution of cellulase to degrade the cell walls (Zimmermann, 2016). However, there was little work for the next 10 years until the commercial enzyme preparations became available for the first time employed commercial enzyme preparation for isolation of protoplasts and subsequently regenerated plants in 1971 (Melchers, 1974; Kao *et al.*, 1990). Protoplasts are now routinely isolated by treating tissues with a mixture of ceil wall degrading enzymes in solution, which contain osmotic stabilizers (Roset and Gilissen, 1989). A general procedure for protoplast isolation has been shown in Fig. 1. The relative ease with which protoplast isolation can be achieved depends upon a variety of factors which have been discussed below (Schell, 1987). (Plate-1-1 Production of Protoplast Isolation and 2 *In vitro* production of isolated plant Table 1.0 *Luffa acutangula* L).

Table 1: Experimental studies on protoplast isolated plants from *Luffa actangula* L.

Growth Regulators (mg/l)	Protoplast isolated plants	Growth response
NAA+0.5 L-Gluranica acid+BAP	35	Callus growth
NAA+1.0 L-Gluranica acid+BAP	30	Green Callus
NAA+2.0 L-Gluranica acid+BAP	25	Green Callus
NAA+3.0 L-Gluranica acid+BAP	20	Small shoot (2-4)
NAA+4.0 L-Gluranica acid+BAP	15	Small shoot bud (3-6)
0.5 BAP+1.0+IBA+ KN	40	Callus growth
1.0 BAP+1.0+IBA+ KN	35	Greening of callus
2.0 BAP+1.0+IBA+ KN	30	Shoot buds (1-2)
3.0 BAP+1.0+IBA+ KN	25	Shoot buds (2-4)
4.0 BAP+1.0+IBA+ KN	20	Profused rooting

Experimental studies on regenerated isolated protoplast plants are transferred to a root inducing medium. In many cases auxin alone or in combination with a low level of cytokinin will enhance root primordial formation in addition to auxin and induction of organogenesis. *In vitro* produced plants in any crops system have to finally reach to the field wher in vivo

conditions. In case of cytoledon calli tha maximum number of shootlets obtaines on MS medium fortified with the combination of BAP, NAA and L-Glutamic acid.

Figure 1: (a)Production of Protoplast Isolation (b)In vitro production of isolated plant

Physiological state of tissue and cell material

Protoplasts have been isolated from a variety of tissues and organs including leaves, petioles, shoot apices, roots, fruits, coleoptiles, hypocotyls, stem, embryos, microspores, callus and cell suspension cultures of a large number of plant species (Fernanda *et al.*, 2000). A convenient and most suitable source of protoplasts is mesophyll tissue from fully expanded leaves of young plants or new shoots. Leaf tissue is popular because it allows the isolation of a large number of relatively uniform cells without the necessity of killing the plants (Bellincampi, 1987; Amberger, 1992). Moreover the mesophyll cells are loosely arranged and enzymes have an easy access to the cell wall (Binding, 1974; Beversdorf, 1977). Leaves are taken; sterilized and lower epidermis from the excised leaves is peeled off. These are cut into small pieces and then preceded for isolation of protoplasts. Since the physiological condition of the source tissue markedly influences the yield and viability of isolated protoplasts, the plants or tissues must be grown under controlled conditions (Butenko *et al.*, 1980; Christianson *et al.*, 1983). Callus tissue and cell suspension cultures used for protoplast isolation should be in the early log or exponential phase of growth. Friable tissue with low starch content generally gives better results.

Enzymes

The release of protoplasts is very much dependent on the nature and composition of enzymes used to digest the cell wall. There are three primary components of the cell wall which have been identified as cellulose, hemicellulose and pectin substances (Cocking, 1960; Duke, 1981). Cellulose and hemicelluloses are the components of primary and secondary structure of cell wall, while pectin is a component of middle lamella that joins the cells. Pectinase mainly degrades the middle lamella, while cellulase and hemicellulase are required to digest the cellulosic and hemicellulosic components of the cell wall respectively. Cellulase (Onozuka) R10 generally used for wall degradation has been partially purified from the molds of *Trichvderma*

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reesei and *T. viride.* Sometimes additional hemicellulase maybe necessary for recalcitrant tissues and for this Rhozyme HP 150 has been used. The most frequently used pectinase is macerozyme (macerase) which was derived from the fungus *Rhizopus.* Driselase, another enzyme, has both cellulolytic and pecteolytic activities and has been successfully used alone for isolation of protoplasts from cultured ceils. Besides, there are several enzymes, e.g. helicase, colonase, cellulysin, glusulase, zymolyase, meicelase, pectolyase, etc. which are used to treat a tissue that does not release protoplasts easily (Table 2).

Table 2: Some commercially available enzymes used for protoplast isolation

Enzyme	Source
Cellulase Onozuka R-10	*Trichoderma viride*
Cellulase Onozuka RS	*Trichoderma viride*
Cellulysin	*Trichoderma viride*
Driselase	*Irpex lacteus*
Meicelase P-1	*Trichoderma viride*
Hemicellulase	*Aspergillus niger*
Hemicellulase H-2125	*Rhizopus* spp.
Rhozyme HP 150	*Aspergillus niger*
Helicase	*Helix pomatia*
Macerase	*Rhizopus arrhizus*
Macerozyme R-10	*Rhizopus arrhizus*
Pectolyase	*Aspergillus japonicus*
Zymolyase	*Arthrobacter luteus*

Aleurone cells of barley treated with cellulase results in protoplasts with the cellulase resistant cell wall (Evans *et al.*, 1983, d'Utra *et al.*, 1992). These cells called sphaeroplasts are to be treated with glusulase to digest the remaining cell wall. The activity of the enzyme is pH dependent and is generally indicated by the manufacturer. However, in practice the pH of enzyme solution is adjusted between 4.7 and 6.0. Generally the temperature of 25~30°C is adequate for isolation of protoplasts. Duration of enzyme treatment is to be determined after trials. However, it may be short for 30-min duration to long duration for 20 h. The enzymatic isolation of protoplasts can be performed in two different ways.

a. **Two-step or sequential method*:*** The tissue is first treated with a macerozyme or pectinase enzyme which separates the cells by degrading the middle lamella. These free cells are then treated with cellulase which releases the protoplasts. In general, the cells are exposed to different enzymes for shorter periods.

b. One step or simultaneous method*:* The tissue is subjected to a mixture of enzymes in a one step reaction which includes both macerozyme and cellulase. The one step method is generally used because it is less labor intensive.

During the enzyme treatment, the protoplasts obtained need to be stabilized because the mechanical barrier of cell wall which offered support has been broken. For this reason an osmoticum is added which prevents the protoplasts from bursting.

Osmoticum

Protoplasts released directly into standard cell culture medium will burst. Hence, in isolating protoplasts, the wall pressure that is mechanically supported by cell wall must be replaced with an appropriate osmotic pressure in the protoplast isolation mixture and also later in the culture medium. Osmotic stress has harmful effects on cell metabolism and growth (Gaj, 2001). A condensation of DNA in cell nuclei and decreased protein synthesis are the two common effects of osmotic stress, although both can be reversed. Lower (more negative) osmotic potentials are usually generated by the addition of various ionic or non-ionic solutes. Non-ionic substances include soluble carbohydrates such as mannitol, sorbitol, glucose, fructose, galactose or .sucrose. Ionic substances are potassium chloride, calcium chloride and magnesium sulphate. Mannitol and sorbitol are the most frequently used with mannitol preferred for isolation of leaf mesophyll protoplasts. Mannitol is considered to be relatively inert metabolically and infuses slowly into the protoplasts.

Mineral salts particularly potassium chloride and calcium chloride has also been used, but there has never been good evidence that they are preferable over mannitol or sorbitol. In general, 0.3 to 0.7 M sugar solution can generate a suitable osmotic potential. Upon transfer of protoplasts to culture medium, it may be appropriate to use metabolically active osmotic stabilizers (e.g. glucose and sucrose) along with metabolically inert osmotic stabilizers, such as mannitol. Active substances will be gradually metabolized by the protoplasts during early growth and cell wall regeneration, resulting in a gradual reduction of the osmoticum. This eliminates sudden changes in osmotic potential when regenerated cells are transferred to a nutrient medium for plant regeneration. In a solution of proper osmolarity, freshly isolated protoplasts appear completely spherical.

Protoplast purification

The enzyme digested mixture obtained at this stage would contain sub-cellular debris, undigested cells, broken protoplasts and healthy protoplasts (Ghazi *et al.,* 1986). This mixture is purified by a combination of filtration, centrifugation and washing. The enzyme solution containing the protoplasts is filtered through a stainless steel or nylon mesh (50-100 µm) to remove larger portions of undigested tissues and cell clumps. The filtered protoplast - enzyme solution is mixed with a suitable volume of sucrose in protoplast suspension medium to give a

final concentration of about 20% and then centrifugation is done at about 100xg for 7-10 min. The debris moves down to the bottom of tube and a band of protoplasts appear at the junction of sucrose and protoplast suspension medium (Gliddie, 1986, 1989). The protoplast band is easily sucked off with a Pasteur pipette into another centrifuge tube. Following the repeated centrifugation and suspension, the protoplasts are washed thrice and finally suspended in the culture medium at an appropriate density. Numerous other gradients have been suggested to aid in protoplast isolation,

- Two step Ficoll (polysucrose) gradients with 6% on top and 9% below, dissolved in MS medium with 7% sorbitol for purification using centrifugation at 150x g for 5 minutes has been suggested. Following centrifugation, debris settles at the bottom while protoplasts float at the top.
- Protoplasts can be separated on to 20% Percoll with 0.25 M mannitol and 0.1 M calcium chloride. After centrifugation at 200x g, protoplasts were recovered above the Percoll. As these high molecular weight substances such as Ficoll and Percoll are osmotically inert, their use may be preferable over sucrose flotation.

Protoplast viability and density

The most frequently used staining methods for assessing protoplast viability are:

1. **Fluorescein diacetate (FDA) staining method***:* FDA, a dye that accumulates inside the plasmalemma of viable protoplasts can be detected by fluorescence microscopy. As FDA accumulates in the cell membrane, viable intact protoplasts fluoresce yellow green within 5 min. FDA is dissolved in acetone and used at a concentration of 0.01 %. Protoplasts treated with FDA must be examined within 5-15 min after staining as FDA disassociates from the membranes after 15 min.
2. **Phenosafranine staining*:*** Phenosafranine is also used at a concentration of 0.01% but it is specific for dead protoplasts that turn red. Viable cells remain unstained by phenosafranine.
3. **Calcofluor White (CFW) staining*:*** Calcofluor White can ascertain the viability of protoplasts by detecting the onset of cell wall formation: CFW binds to the beta- linked glucosides in the newly synthesized cell wall which is observed as a ring of fluorescence around the plasma membrane. Optimum staining is achieved when 0.1 ml of protoplasts is mixed with 5.0 pi of a 0.1% w/v solution of CFW.
4. Exclusion of Evans blue dye by intact membranes,
5. Observations on cyclosis or protoplast streaming as a measure of active metabolism.
6. Variation of protoplast size with osmotic changes,
7. Oxygen uptake measured by an oxygen electrode which indicates respiratory metabolism,
8. Photosynthetic studies.

However, the true test of protoplast viability is the ability of protoplasts to undergo continued mitotic divisions and regenerate plants. Protoplasts have both maximum as well as minimum plating densities for growth. Published procedures suggest that protoplasts should be cultured at a density of 5 x 10^3 to 10^6 cells/ml with an optimum of about 5 x 10^4 protoplasts/ ml (Hansen, 1999). The concentration of protoplasts in a given preparation can be determined by the use of hemocytometer.

A. Culture techniques

Isolated protoplasts are usually cultured in either liquid or semisolid agar media plates. Protoplasts are sometimes allowed to regenerate cell wall in liquid culture before they are transferred to agar media.

B. Agar culture

Agar of different qualities is available but agarose is most frequently used to solidify the culture media. Bergmann cell plating technique as explained earlier is followed for plating of protoplasts. Protoplast suspension at double the required final density is gently mixed with an equal volume of double the agar concentration in the medium kept molten at 45°C. The concentration of agar should be chosen to give a soft agar gel when mixed with the protoplast suspension. The petri dishes are sealed with parafilm and incubated upside down. By agar culture method, protoplasts remain in a fixed position so that protoplast clumping is avoided. Protoplasts immobilized in semisolid media give rise to cell clones and allow accurate determination of plating efficiency. Once immobilized, however, hand manipulations are required for transfer to other culture media.

C. Liquid culture

It has been generally preferred in earlier stages of culture because (i) It allows easy dilution and transfer; (ii) Protoplasts of some species do not divide in agarified media; (iii) Osmotic pressure of the medium can be effectively reduced; (iv) Density of cells can be reduced after few days of culture. But it has the disadvantage of not permitting isolation of single colonies derived from one parent cell. Various modifications to these culture methods have been developed.

D. Liquid droplet method

It involves suspending protoplasts in culture media and pipetting 100-200 pi droplets into 60 x 15 ml plastic petri dishes. Five to seven drops can be cultured per plate. The plates are then sealed and incubated. This method is convenient for microscopic examination, and fresh media can be added directly to the developing suspensions at 5-7 days interval. A problem encountered in this method is that sometimes the cultured protoplasts clump together at the center of the droplet.

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E. Hanging droplet method

Small drops (40-100 µl) of protoplast suspension are placed on the inner side of the lid of a petri dish. When the lid is applied to the dish, the culture drops are hanging or suspended from the lid. This method allows culture of fewer protoplasts per droplet than the conventional droplet technique.

F. Feeder layer

In many cases it is desirable to reduce the plating density to a minimum for a given protoplast preparation. A feeder layer consisting of X-irradiated non-dividing but living protoplasts are plated in agar medium in petri dishes. Protoplasts are plated on this feeder layer at low density in a thin layer of agar medium. This is especially important when particular mutant or hybrid cells are to be selected on agar plates.

G. Co-culturing

It is the culturing of two types of protoplasts viz. slow growing and fast growing. A reliable fast growing protoplast preparation is mixed in varying ratios with protoplasts of a slow growing recalcitrant species. The fast growing protoplasts presumably provide the other species with growth factors and undefined diffusable chemicals, which aid the regeneration of a cell wall and cell division.

H. Culture medium

Protoplasts have nutritional requirements similar to those of cultured plant cells. The mineral salt-compositions established for plant cell cultures have been modified to meet particular requirements by protoplasts. Protoplast culture medium should be devoid of ammonium as it is detrimental to its survival and quantity of iron and zinc should be reduced. On the other hand calcium concentration should be increased 2-4 times over the concentration normally used for cell cultures. Increased calcium concentration may be important for membrane stability. Mannitol and sorbitol are the most frequently used compounds for maintaining the osmolarity, which has already been discussed. Glucose is perhaps the preferred and most reliable carbon source. Plant cells grow about equally well on a combination of glucose and sucrose, but sucrose alone may not always be satisfactory for plant protoplasts. Henry *et al.,* (1998). The vitamins used for protoplast culture include those present in standard tissue culture media. If protoplasts are to be cultured at very low density in defined media there may be a requirement for additional vitamins. Protoplast media frequently contain one or more amino acids. A convenient approach is to add 0.01 to 0.25% vitamin-free casamino acids or casein hydrolysate. Such mixtures appear to meet the needs of protoplasts if inorganic nitrogen is inadequate. The addition of 1-5 mM L-glutamine can improve growth.

The majority of protoplast culture media contain one or more auxins plus one or two cytokinins to stimulate protoplast division and growth. Only a few protoplast systems grow

without added growth regulators in the cultures media. In general, protoplast culturing starts with a relatively high concentration 1-3 mg/l of NAA or2,4-D along with a lower concentration (0.1 to 1.0 mg/l) of BAP or zeatin. When protoplast division has started, it is often recommended to change the exogenous hormone supply. A change in the auxin to cytokinin balance is often used for stimulating morphogenesis.

I. Environmental factors

Generally high light intensity inhibits protoplast growth when applied from the beginning of culture. It is better to initiate protoplast culturing in darkness or dim light for few days and later transfer the cultures to a light of about 2000 -5000 lux. There are reports of better protoplast growth when the cultures are kept in continuous darkness. In contrast, it has been shown for legume species that light is necessary for initiating protoplast division. Protoplast cultures are generally cultured at temperatures ranging between 20-28°C. A pH in the range of 5.5 to 5.9 is recommended for protoplast culture media and it seems to be satisfactory.

Conclusion:

Protoplasts in culture generally start to regenerate a cell wall within a few hours after isolation and may take two to several days to complete the process under suitable conditions. The protoplasts lose their characteristic spherical shape, which is an indication of new wall regeneration. However, newly synthesized cell wall by protoplasts can be demonstrated by staining it with 0.1 % calcofluor white fluorescent stain. In general, protoplasts from actively growing suspension cultures exhibit more rapid deposition of microfibrils than those from differentiated mesophyll cells. A freshly formed cell wall is composed of loosely arranged microfibrils which subsequently become organized to form a typical cell wall. Macroscopic colonies are transferred to an osmotic free medium to develop callus. The callus may then be induced to undergo organogenic or embryogenic differentiation leading to the formation of plants. The first report of plant regeneration was since then the list of species exhibiting the totipotency has steadily increased. Several reports have suggested that frequency of plant regeneration reported from calluses derived from plant organs differ from the calluses raised from protoplasts. The calluses from intact plant organs often carry preformed buds or 'Organized structures, while such structures are absent in the callus from protoplast origin.

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AN UPDATED OVERVIEW ON TOPICAL DRUG DELIVERY SYSTEMS

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Abstract:

The paper is aimed to supply a comprehensive review on the traditional and novel approaches for delivering the drug to skin through transdermal route. Transdermal route of drug delivery within the era transdermal route of drug delivery has gained popularity over other methods. Various dosage forms are available for transdermal delivery of drug like creams, poultices, ointements, paste, jellies, transdermal patches, emulgels to call a couple of. Main advantage of TDDS is that it reduces the load placed commonly by oral route on alimentary canal and liver. Patient compliance is increased and also harmful side effects are decreased. TDDS offer improve bioavailability, uniform plasma levels, longer duration of action. The main difference between oral dosage forms and TDDS is that there's decline in plasma level in oral dosage forms but with TDDS plasma levels are improved. Various other advantages of TDDS are limitations of first Pass metabolism, increase in therapeutic efficacy, steady plasma levels are maintained.

Keywords: TDDS, Skin

Introduction:

Structure of skin:

The Skin is that the largest organ of physical body having an area of just about 2 square feet. Skin is that the protective barriers which supply protection against microbes and elements and helps in regulation of blood heat.

Skin is anatomically divided into four layers –

1. Epidermis (outer layer)
2. Dermis (tough animal tissue and sebaceous glands)

3. Corneum

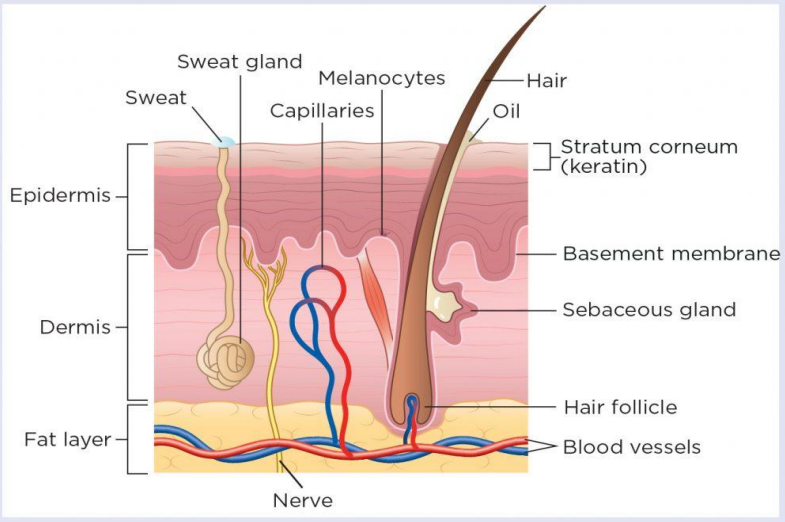
4. Hypodermis (made of fat and connective tissue)

Epidermis:

Epidermis is that the self renewing stratified squalors epithelium covering the outer surface of body. Keratinocytes are the main constituent of epidermis. Protein keratin is produced by Keratinocytes which are the main building blocks (cells) of the epidermis. Because the epidermis is avascular (contains no blood vessels), it's entirely hooked in to the underlying dermis for nutrient delivery and waste disposal through the basement membrane

The prime function of the epidermis is to act as a physical and biological barrier to the external environment, preventing penetration by irritants and allergens. At an equivalent time, it prevents the loss of water and maintains internal homeostasis. Keratinocytes structure around 95% of the skin cell population – the others being melanocytes, Langerhans cells and Merkel cells [1, 2].

Fig 1. **Cross-section through the skin**



Dermis:

The dermis is that the inner layer of the skin and is anatomically much thicker than the epidermis (1-5mm).Situated between the basement membrane zone and therefore the subcutaneous layer, the first role of the dermis is to sustain and support the epidermis. the most functions of the dermis are:

- Protection.
- Cushioning the deeper structures from mechanical injury;

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- Providing nourishment to the epidermis;
- Playing a crucial role in wound healing.

Hypodermis:

The hypodermis is that the subcutaneous layer lying below the dermis; it consists largely of fat. It provides the most structural support for the skin, also as insulating the body from cold and aiding shock absorption. it's interlaced with blood vessels and nerves.[3]

Desirable characteristics of topical drug:

Delivery systems [2, 3]

1. To assist in hydrating the skin due to their emollient action.
2. To guard from external environment or heal an intact or injured area of the skin.
3. To deliver medication to the skin.

TDDS dosage forms:

1. Creams:

The cream is that the TDDS which is taken into account as a crucial part in both cosmetics and pharmaceutical due to easy application and straightforward removal. It categorized under the pharmaceutical product and is ready by various techniques which are developed by the pharmaceutical industry, are by mixing the two-phase (O & W) phase to make a cream then evaluated by employing a various evaluation process e.g. of pH, viscosity, spreadability, stability study. The cream could also be classified as an o/w and w/o sort of emulsion [3, 4].

Skin cream is assessed supported –

1. Function e.g. cleansing, foundation, massage, etc.
2. Characteristics properties, e.g. cold creams, vanishing creams, etc.
3. Nature or sort of emulsion.

Types of topical creams): -

- Make – up creams (o/w emulsions)

1. Vanishing cream
2. Foundation cream

- Cleansing cream (w/o)
- Cream for winter (w/o)

1. Cold cream

• Cream for dry skin

1. Moisturizing cream

• All-purpose creams

• Night creams

• Skin protective and cream.

Advantages and Disadvantages of cream as a topical drug delivery system [5,6].

1. Avoidances of the first-pass metabolism.
2. Avoid risk.
3. Convenient and straightforward to use.
4. It doesn't show the side effect on the opposite body organ.
5. Easy termination of medicines, when needed.
6. Avoid alteration of drug levels inter-and intra- patent variations.

Disadvantages:

1. Skin irritation
2. Some drugs show low penetrable through the skin.
3. Possibility of allergies.
4. Small plasma concentration.
6. Larger particle size drug is showing the poor effect.

2. Emulgels:

Emulgel is emulsions, both of the water-in-oil or oil-in-water type, which is gelled by blending in with a gelling specialist Emulgels have a couple of free properties for dermatological utilize, for instance, being thixotropic, greaseless, effectively spreadable, with none problem removable, emollient, non-recoloring, while span of usability, biofriendly, straightforward and satisfying appearance.[7,5]

Advantages:

1. Evasion of first-pass digestion.
2. Evasion of gastrointestinal contradiction.
3. More particular to a specific site.
4. Improve tolerant consistency.
5. Appropriateness for self-prescription.
6. Giving use of medication short natural half-life and restricted restorative window.
7. Capacity to handily end medicine when required.
8. Advantageous and straightforward to use.

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9. Joining of hydrophobic medications
10. Better stacking limit.
11. Better soundness.
12. Creation attainability and low planning cost
13. Controlled delivery.

3. Ointment:

Ointments are homogenous, semi-solid preparations intended for external application to the skin or mucosa. They're used as emollients or for the appliance of active ingredients to the skin for protective, therapeutic, or prophylactic purpose and where a degree of occlusion is desired.[8,9]

Hydrophobic Ointments:

Hydrophobic (lipophilic) ointments are usually anhydrous and may absorb only small amounts of water. Typical bases used for his or her formulation are water-insoluble hydrocarbons like hard, soft and liquid paraffin, oil, animal fats, waxes, synthetic glycerides, and polyalkylsiloxanes.

Water-Emulsifying Ointments:

Water-emulsifying ointments can absorb large amounts of water. They typically contains a hydrophobic fatty base during which a w/o agent, like lanolin, wool alcohols, sorbitan esters, monoglycerides, or fatty alcohols are often incorporated to render them hydrophilic. they'll even be w/o emulsions that allow additional quantities of aqueous solutions to be incorporated. Such ointments are used especially when formulating aqueous liquids or solutions.

Hydrophilic Ointments:

Hydrophilic ointment bases are miscible with water. The bases are usually a mix of liquid and solid polyethylene glycols (macrogols)

4. Gels:

The U.S.P. defines gels as a semisolid system consisting of dispersion made from either small inorganic particle or large organic molecule enclosing and interpenetrated by liquid. They contain a two component semi-solid system rich in liquid. Their one characteristic feature is that the presences of continuous structure providing solid like properties. Gels became a premier

materials used for drug delivery formulations thanks to its biocompatibility, network structure, and molecular stability of the incorporated bioactive agent.

5. Pastes:

Pastes, like ointments, are intended for external application to the skin. They differ from ointments primarily therein they typically contain a bigger percentage of solid material and as a consequence are thicker and stiffer than ointments thanks to their large percentage of solids. Pastes are generally more absorptive and fewer greasy than ointments prepared with an equivalent components. thanks to stiffness and absorptive qualities of paste, they still be in place after application with little tendency to melt and flow. Therefore these are effectively employed to take in serous secretions from the location of application. Pastes are therefore preferred over ointments for acute lesions that have a bent toward crusting, vesiculation or oozing. However, thanks to their stiffness and impenetrability, pastes aren't generally fitted to application to hairy a neighborhood of the body.[9,10]

Example: zinc-oxide paste

Newer research in topical drug delivery systems [11, 12]:

Enzyosomes:

Enzymosomes is an innovative, currently emerging targeted vesicular drug delivery system. Enzymosomes uses enzymes, which are having a targeted catalytic function for a substrate, which are in corporate within cell-like structures having a high lipid background. It yields newly designed liposomes during which the enzymes are coupled covalently to the surface of lipid molecules.Enzymes links through acylation, direct conjugation, physical adsorption, encapsulation methods to arrange enzymosomes with targeted action. Such novel drug delivery systems prove effective drug release and concomitantly reduce undesirable side effects of conventional treatment methods and hence showcase improvement within the long-term therapy of the disease. They seem to be a promising substitute to plain treatment therapies of gout, antiplatelet activities etc. Enzymosomes are newly designed supramolecular vesicular delivery systems to be useful as a tool in pharmaceutics for the raising of drug targeting and physicochemical properties and hence bioavailability

Ethosomes:

Ethosomes are non invasive method of delivering vesicular carrier composed mainly of phospholipids, alcohols, and water. It allows drugs to penetrate the deep layers of skin or

circulation. Although ethosomal systems are conceptually sophisticated, they're characterized by simplicity in their preparation, safety, and efficacy a mixture which will highly expand their application.

Erythrosomes:

Erythrosomes are mechanically stable large proteoliposomes. This are cross-linked erythrocyte cytoskeletons, which supports to the coated lipid bilayer effective in targeting of macromolecular drugs.

Conclusion:

The present review has focused on novel also as conventional topical drug delivery systems. TDDS are gaining attention over other dosage forms over the last century. However it can't be denied that conventional drug delivery systems suffer from lot of drawbacks. On contrast Novel Topical drug delivery systems have several profound advantages like better patient compliance, safety, efficacy, feasibility and time period.So it are often undoubtly concluded that converting the traditional formulation into novel formulation by using carriers will pave a replacement path within the treatment of varied diseases.

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SIGNIFICANCE OF RED ALGAE IN TERMS OF FOOD AND MEDICINE

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Introduction:

The red seaweed is a group of eukaryotic algae from the class Rhodophyceae of the group Rhodophyta. These algae are found in marine water of sea and ocean. The red algae constitute of phycoerythrin and phycocyanin pigments .The red pigment impart peculiar red colour to thallus. The thallus consists of hold fast, stipe and fronds or leaves .The thallus is small and beautiful in appearance. The reserve food material in these algae is floridian starch. The members of red algae reproduce by both sexual and asexual means. The sexual reproduction takes place by specialized male structure called Spermatia and female structure Carpogonia .The seaweeds are significantly important for man and animals. The use of seaweeds is traced back in many countries like Japan, Korea, Europe, USA, Philippines, China etc. The people those lived near the coastal area have preferred to consume seaweeds as a part of diet .The proportion of intake of seaweed has been gradually increased due to food value and medicinal significance due to bioactive compounds .The public awareness has enhanced tremendously regarding the use of seaweeds. The utility of seaweeds has encouraged the domestic utilization and commercial marketing. The commercialization is made by harvesting the seaweeds through mariculture, land-based culture and seaweed farming.

The red seaweeds algae are rich source of bioactive compounds like protein, sulphated polysaccharides, pigments, polyunsaturated fatty acids, vitamins, minerals and phenolic compounds. The red algae are used due to nutritious and medicinal value. The Polysaccharides in the cell wall is extensively utilized in pharmaceutical industries. The polysaccharide has thickening and gelling property. The hydrocolloids, galactans, carrageenans, agars are the cell wall polysaccharides applied therapeutic use .The chemical constituents are regulators sugar . They are nutrient enhancers to the body .The Red algae forms an important part of the marine ecosystem as it is used as food aquatic and terrestrial animals. The aquatic animals like zooplanktons, fishes, crustaceans, worms are eating on the seaweed. The red algae are used as fodder for livestock. The red algae have significance as food and medicine.

There are some genera of red seaweeds or red algae such as *Porphyra, Chondrus, Rhodymenia, Gelidium, Gracilaria, Gloiopeltis etc.* They are significant for man and animals.

1. Porphyra

Occurrence:

It is a red alga commonly called as red seaweed. It is also recognized by the name Laver. It grows in marine water of sea and ocean .It is generally found grown in intertidal zone.

Food value:

This alga is commercially cultivated in many countries of the world such as Korea, China, USA, Europe, Vietnam, Japan etc. There are various species of *Porphyra* that are widely cultivated as marine crop. The thallus contains mineral nutrients, proteins, carbohydrates, vitamins, iron and fatty acids. The thallus of *Porphyra* is cultivated, harvested, dried, processed and consumed or marketed all over the world .The *Porphyra tenera* and *Porphyra yezoensis* are used to produce dry and commercial food products , they are called as dried seasoned laver and roasted laver .The dried and roasted laver are popular in global market as food products .It is a priced food due to its texture, compactness, pleasant taste and high nutritional value. The seaweed of red algae is used to wrap the rice and fish .The food prepared from *Porphyra* is known as Sushi in Japan, Gimbap in Korea and Limu lua in Hawaii Island. The *Porphyra umbilicalis contains* high amount of proteins. The proteins are used as a potential ingredient in meat products and enhance nutritional value of food. The *Porphyra* also contains different types of amino acids such as Serine, Glycine, Alanine, Valine, Tyrosine, Phenylalanine and Arginine.

Medicinal value:

The *Porphyra* contains many bioactive compounds which are used in preparation of medicines. The bioactive compounds enrich the health of human. The thallus contains polysaccharides, phycobiliproteins and peptides. These compounds are used in the preparation of pharmaceutical products. The sulfated galactan polysaccharide significantly decreases the lipid and helps for proper functioning of cardiovascular system.

2. Chondrus

Occurrence:

It is a marine red alga found in marine water of sea and ocean. It grows rock substratum in the subtidal to middle intertidal zone. It produces dichotomous branches. There are many species of *Chondrus* like *Chondrus crispus*, *C. ocellatus*, *C. nipponicus*, *C. yendoi*, *C. pinnulatus* and *C. armatus*.

Food value:

The species of *Chondrus* are widely harvested and used as a sea vegetable food. It yields Carrageenan substance which is having gelling property. The carrageen is used as a thickener and stabilizer in preparation of milk products especially ice cream and chocolate milk.

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Medicinal value:

This alga is rich in potassium chloride contents. It has anti-bacterial and anti-viral properties. The bioactive compounds present in thallus are good for dry skin, so it can be used as skin conditioning and to cure psoriasis. It is used in making lotions and moisturizers. The hydrophilic molecules provide hydration to protect and soften the skin. The presence of fatty acids and phenols helps to preserve and protect the natural skin, as it reduce dryness of skin.

3. Rhodymenia

Occurrence:

It is a red marine alga found in marine water attached to rock substratum. This alga is commonly called as dulse or dillisk or dilsk or Sheep weed. This genus is also identified with the name *Palmaria palmate*. It is called as red dulse or sea lettuce flakes. It was previously referred to as *Rhodymenia palmata*. The thallus consisted of short stipe and broad red-tinted fronds or leaves.

Food value:

The thallus contains mineral nutrients, fibres, vitamins and proteins. The fronds or leaves are collected, dried and grinded and used as ingredients in food and medicine preparation. The dulse can be used as flavouring agent in food, soups and salads. It is a popular snack food. The thallus is used as fodder for cattles. It can be eaten directly or after drying. The thallus can be cut into pieces and pan-fried into chips; it can be baked with cheese and eaten. The thallus can be used in preparation of soups, sandwiches, salads, bread and pizza.

Medicinal value:

The thallus constitutes of calcium, magnesium, potassium, Iodine and iron. These minerals enhance density of bones and enrich the joints and other tissues of bones. It makes the bones stronger. The osteoporosis of bones can be improved through the use of dulse. The Potassium found in leaves is a vasodilator which reduces the strain on blood vessels and arteries during high blood pressure. It helps to lower blood pressure and protects coronary from heart disease, strokes and heart attacks. The Potassium helps to increase blood flow to the brain and capillaries, so it is considered as brain tonic. The thallus contains vitamin A, Vitamin B-12, Vitamin B6. The vitamins help to build and maintain health of tissues, teeth, bones, eyes and skin. The Vitamin A cures vision problems of eyes. The antioxidants in thallus prevents free radicals that damaging the tissues of eye. It boost eye vision and reduces the chances of cataract. The vitamin C increases white blood cell which enriches the immune system of body. The deficiency of iodine causes goitre disease. The iodine contents in thallus help to keep thyroid gland normal and avoid thyroid disorder. The iron in thallus is useful in the production of haemoglobin. The intake of dulse enriches the level of haemoglobin in blood and enhances

blood circulation. The anaemia disease can be cured with intake of dulse in food. The leaves contain Omega-3 and Omega-6 polyunsaturated fatty acids. These omega acids are important against blood clotting and arthritis. The fatty acids improves the functioning of brain and nervous system. The fibres present in the leaves helps to regulate digestive process.

4. Gelidium

Occurrence:

It is a red alga found in sea and oceans. It is found attached with the rocky substratum. It can be harvested from shallow coast. The *Gelidium* is also called as *Chaetangium*.

Food value:

The thallus of *Gelidium amansii* contains plenty of agar substance. The agar can be used to reduce the weight of body. The agar constitutes of polysaccharides like agarose and agaropectin. The agar can be used as an ingredient in preparation of salad, puddings, jams and food dishes. The thallus is a rich source of carbohydrates like galactose and glucose. Due to gelling property of agar it is used in making gelatine capsules and material which is used in making dental impressions as well as base for cosmetics. The agar is used as a nutrient food for culture of bacteria, fungi and other microorganisms. It can be used as a preservative for meat and fish. The agar is also used in making dairy products like ice creams.

Medicinal value:

The thallus yield agar is used to reduce greedy habit of eating. The agar contains fat, sugar and carbohydrates. It gives feeling of full eating by avoiding excess food. The agar absorbs glucose in stomach and inhibits storage of fat. The use of teaspoon of agar powder in tea or hot water before meal gives good result of weight loss. To obesity can be reduced through the use of agar in diet. The agar helps to lower food calories. The fibres in the agar absorb toxins from the gut and gastrointestinal tract and propel toxic waste out of body. The agar can be used as a remedy against constipation. If the faecal content is hard, dry and painful, then agar is used for normal functioning of gut and rectal region. The hard stool causes pressure on the rectum then fissures can be formed. The agar contains high amount of calcium and magnesium. The calcium is useful in making bones and teeth strong. The magnesium increases density of bones. The galactose of agar is considered as brain sugar which is vital for development of brain. It is a component of myelin sheath which protects brain, spinal cord and central nervous system. The galactose can be used to remove neurotoxic chemicals from the brain. The patients suffering from alzheimer's disease can be cured through the application of agar. The agar decreases the cholesterol in blood and saves a person from heart disease and attack.

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5. Gracilaria

Occurrence:

It is a red alga found in marine water. It is found attached with the rock substratum. The thallus is branched and beautiful in appearance.

Food value:

The species of *Gracilaria* are used as a food in many countries like Korea, Philippines, Jamaika, China, and Japan etc. In Japan the seafood made from this alga is called as ogonori or ogo. In Philippines it is called as gulaman. In Jamaica it is known as Irish moss. In Korea it is known as kosiraegi. The thallus of *Gracilaria* contains sulphated oligosaccharides. This alga obtained from this alga is used in making food dishes. *Gracilaria verrucosa* is valuable as food in Korea. The agar of this alga is used in making dairy products and chocolates.

Medicinal value:

The bioactive compounds in thallus are used in the treatment of muscle inflammation and gastric complaints. The galactose and glucose are the important chemical constituents of this alga. The thallus yields a non-nitrogenous substance agar, which is have gelling property. The gelling is useful in making gelatine capsules and dental impressions. The agar is useful in preparation of anti-inflammatory and anti-ulcerogenic drugs.

6. Gloiopeltis

Occurrence:

It is a red alga found in water of sea and ocean. This alga is Glue weed, Jelly moss and Fukuro-funori.

Food value:

The thallus of this alga is used as a food source for man and animals. *Gloiopeltis tenax* is important edible seaweed which is used as an ingredient in food. The thallus contains mineral nutrients and vitamins. The nutrient value enhances the necessity in diet.

Medicinal value:

The thallus extract of *Gloiopeltis tenax* is used in the treatment of diarrhoea and Colitis. The extracts of *Gloiopeltis furcata* is used to inhibit the growth of cancer cells. It reduces high blood sugar or glucose level.

7. Gracilariopsis

Occurrence:

It is a marine red alga found in seas and ocean. It grows in marine water with support of rock.

Food value:

The thallus of *Gracilariopsis chorda* is used as a food ingredient due to rich mineral and vitamin contents. It yields agar which is used in preparation of various food products.

Medicinal value:

The thalluses possess antioxidant and anticancer compounds. The bioactive compounds are in inhibition of cancer cells. The anti-inflammatory substance is produced from the extract of thallus.

The properties of Red Seaweed:

i) It serves as an antioxidant:

The free radicals are produced during metabolism at the time of inflammatory responses or phagocytises or physical exercise. The excess amount of free radicals damages proteins, amino acids, nucleic acids and carbohydrates in human body. The oxidative damage to tissues can be inhibited and reduced by the use of red seaweeds due to antioxidant property.

ii) It has an anticancer potential:

The cancer is the major health problem of many people. The cancer causes large number of death all over the world. The bioactive compounds and metabolites present in red seaweed enable to suppress excessive growth of cancer cells and reduce the risk of cancer development.

iii) It serves as an anti-inflammation agent:

The continuous inflammatory responses are harmful to living tissues of human body. The inflammation causes destruction of tissues and promotes cancer. The extract of red seaweed contains anti-inflammatory compounds. The use of thallus in diet reduce inflammatory effect.

iv) It serves as an anti-diabetic agent:

The diabetes is a prevalent and chronic metabolic disease in many people. It is an ailment in body which alerts the level of glucose due to abnormal functioning of metabolism. In diabetic patients glucose level is increased which affects the normal body functions. The bioactive compounds and chemical constituents in thallus reduce and maintain normal sugar level in body.

v) It serve as an anti-obesity agent:

The obesity is a metabolic disorder in human being. The obesity is increased due to excessive accumulation of fat in body. In this disorder there is an increase of weight that causes obesity. The red seaweeds contain mineral nutrients and vitamins. The nutrient of thallus helps to avoid excess eating. The eating of seaweed ingredients reduces appetite of a person which result in loss of weight and obesity. Thus bioactive compounds help to minimize initiation of diabetes, cardiovascular and renal diseases.

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vi) It serves as anti-coagulant agent:

The red seaweed contains valuable secondary metabolites which functions as anticoagulant agents. The anticoagulant agents are used in the treatment of alopecia, skin allergic reactions, eosinophilia and osteoporosis.

Conclusion:

The red seaweeds are valuable source of food and medicines. They are used due to nutritious and medicinal property. The seaweed food is significant and important to man and animals. The sea weeds are utilized in many countries of the world. The thallus of red algae are used in food and pharmaceutical industries. The secondary metabolites present in seaweeds are potentially useful. The products obtained from seaweeds are used for domestic and commercial purpose all over the world. The thallus of red algae yields sulphated galactans such as agar, agarose and carrageenans substances. These substances are the polysaccharides. They have ability to form strong gels in aqueous solution. The gelling property helps to in preparation of various food, medicine and cosmetic products. The red algae is a renewable source of energy for sustainable development.

Future Perspective:

The red algae have attracted considerable interest worldwide because of its significance in food and medicines. The food need of ever increasing human population can be fulfilled through the seaweeds. The seaweeds can be an alternative food source for animals. The food, fuel and medicines are valuable in human life. The nutrition value of diet can be enhanced with bioactive compound in thallus. The fuel source of energy can be fulfilled through biodiesel and bioethanol that obtained from thallus of red seaweeds. The biofuels are ecofriendly solution that will help to reduce air pollution and global warming. The cost-effective algal produce depends upon the improvement and optimization of culturing and harvesting techniques. The human and animal diseases can be treated and cured by the use of bioactive compounds present in thallus.

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ANTI-ARTHRITIC ACTIVITY OF SOME MEDICINAL PLANTS – A REVIEW

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Abstract:

Arthritis is an inflammation of joints which causes severe pain and deformity in joints due to release of pro-inflammatory markers, cytokines and leukotrines. The osteoarthritis and rheumatoid arthritis is the two most prevalent types of arthritis. The cause for Rheumatoid arthritis includes genetic factor, family history, age, environmental factors, hormones, smoking. Synthetic drugs are used for the better improvement from the symptoms, but they offer only temporary relief and produce severe side effects. Hence researchers are concentrating on natural remedies, for treatment of various diseases, with efficacy and safety, and with fewer side effects. In this paper a few of the medicinal plants with their phytoconstituents that can be used in the treatment of RA (Anti-arthritis) and different treatments available for Rheumatoid arthritis is discussed which can be explored for further studies.

Keywords: **A**rthritis, synthetic drugs, natural remedies, bioactive constituents, rheumatoid arthritis, treatment.

Introduction:

Arthritis is the swelling (inflammation) of joints and tenderness of one or more of joints. This causes mild to severe pain, stiffness, redness or heat in joints with decreased physical motion and inability which typically worsen with age. Thus Arthritis is considered to be a sign or a symptom. The joints are places that will have the bones coming together like knee, wrist, fingers, toe and hip region. Arthritis is threatening that it results in deformity of the joints permanently. They also affect the ligaments, bones, tendons and muscles. The osteoarthritis and rheumatoid arthritis is the two common among the arthritis. Osteoarthritis causes the breakdown of cartilage; the tissue that covers the bone end where joint is formed. Rheumatoid arthritis (RA) is an autoimmune disease, in which the joints are attacked by the immune system, beginning with the lining of joints. In rheumatoid arthritis lumps on their skin called rheumatoid nodules are formed in certain cases. Gout is caused due to uric acid crystals that form when there's too

much uric acid in blood. Infections or underlying disease, such as psoriasis or lupus, can cause other types of arthritis. Hence there are 100 rheumatic diseases that affect nearly millions of people worldwide. Especially in India 15% of the population (180 million people) is affected by this arthritis [1]. RA mainly affects women than men in ratio of (3:1) generally, striking between 30 and 55 years [2].

The etiology of RA is not known precisely, but a genetic susceptibility in combination with the environmental factors influence for the onset of RA. Some of the common factors include:

- Environmental factors.
- Silica exposure.
- Diet.
- Blood transfusion.
- Impact of sex and sex hormones especially during pregnancy, postpartum period and menopause [3].
- Genetic factors have an impeccable impact as the disease can run in families. People who have specific human leukocyte antigen (HLA) genes have more chance of developing rheumatoid arthritis [4].

Rheumatoid arthritis primarily starts in synovium, producing a sac surrounding the joint that contain the synovial fluid lubricating and cushioning to provide support and flexibility to the joints. They supply nutrients and oxygen to cartilage coating the end of bones that is made of collagen. Abnormal immune response is responsible for inflammation of the synovium where the collagen destruction accelerates damaging the bone by narrowing the joint space. In a progressive rheumatoid arthritis pannus (thickened synovial tissue) formation is seen due to the accumulation of fluid and immune system cells in the synovium. It attracts more inflammatory white cells, B cell and T cell lymphocytes that are important in inflammation associated with rheumatoid arthritis. "Non-self" antigens are recognised by T cells and it will produce chemicals (cytokines) which stimulate B cells to multiply and release antibodies into the bloodstream, triggering inflammation against foreign particles invasion [5]. Pro-inflammatory markers, cytokines and leukotrines presence is the cause of RA. The IL-1, TNF-α, IL-6, IL-15, IL-16, IL-17, IL-18, IFN-γ, and granulocyte macrophage-colony stimulating factor, chemokines such as IL-8, macrophage inflammatory protein-1 and monocyte chemo attractant protein-1are primary inflammatory markers [6].

Primarily the treatment is focused on decreasing the disease activity or the inflamed condition, with a minimal of joint destruction. The treatment for the disease includes;

- Non-steroidal anti-inflammatory drugs (NSAIDs), which lessen pain and stiffness [7].
- Corticosteroids.
- Disease modifying anti-rheumatic drugs (DMARDs), can reduce joint swelling and pain, decrease acute phase markers and also limit progressive joint damage and improve function.
- Biological agents, involves TNF-α blockade, IL-1 blockade, B cells therapy, IL-6blockade and Angiogenesis blockade.

Treatments differ depending on the type of arthritis [8]. Thus the arthritis treatments are carried out to reduce symptoms and improve quality of life. Hence there are different treatments available for arthritis like NSAIDs, Steroids, etc. These treatments can relieve pain, and the disease can be controlled to a certain extent, with severe side effects [9]. Medicinal plants have been extensively used as major sources of cure to human diseases since time immemorial. Also today, one fourth of world population depends on traditional medicine and 80% of the population relies on indigenous medicinal plants [10]. Hence this review has explored a research on some common plants for the anti-arthritic activity.

Medicinal plants possessing anti-arthritic activity:

Moringa oleifera

The drumstick tree, *Moringa oleifera*, is a well-known and inexpensive source of phytochemicals with potential medical applications. Glucosinolates, flavonoids, phenolic acids, carotenoids, tocopherols, polyunsaturated fatty acids, highly bioavailable minerals, and folate are the main phytochemical constituents [11]. The disease model of Complete Freund's Adjuvant induced arthritis in rats was used to assess anti-arthritic function [12]. According to the analysis of different arthritic evaluation criteria used in this report, moringa extract plays a significant role in preventing the development or ameliorating the severity of arthritis disease.Anti-inflammatory and antioxidant activity of *Moringa* leaves extract indicated a significant inhibition of nitric oxide (NO) production by macrophage cells, decrease in serum level of IL-1, IL-6, and TNF-a in addition to inhibition of COX2 pathway by inhibition of PGE2 production. This finding suggests that Moringa extract effectively decreases the inflammatory-associated pain, and not only central action but also peripheral inhibition of the prostaglandins-mediated potential of analgesic action of bradykinins were involved. Methanol extract of combined roots and leaves

of Moringa in three arthritic models in rats with an ethanol extract of Moringa leaves in a collagen-induced RA [13].

Trichosanthes dioca

The cucumber and squash-like vine *Trichosanthes dioca*, also known as pointed gourd, belongs to the *Cucurbitaceae* family. It is chock-full of vitamins and minerals. Phytochemical constituents include flavonoids, tannins, phenols, alkaloids, and glycosides, as well as fats and carbohydrates.Immunologically mediated FCA induced arthritic model is considered for ongoing study. Polyphenol content and presence of tannins also reveals anti-inflammatory action of *Trichosanthes diocia*. Prevalent inhibition of lipid peroxidation and enzyme activity like cyclooxygenase, lipoxygenase due to tannins and polyphenols components was observed. Thus, resulting immunomodulation effect indicates the antiarthritic activity of Hydroalcoholic extract of *Trichosanthes diocia*. In complete Ferund's Adjuvant induced arthritic models, hydro alcoholic extracts showed moderate anti-arthritic activity [14].

Skimmia anquetilia

The *Rutaceae* family includes the aromatic gregarious shrub *Skimmia anquetilia.* It is mostly found in Kashmir and the Western Himalayas of India. Since HRBC membrane are like lysomal membrane components, the prevention of hypotoxicity induces HRBC membrane lysis is taken as a measure of anti-inflammatory activity of drugs. Hence the ethyl acetate extract of *S. anquetilia* showed significant anti-inflammatory activity at the concentration of 400 mg/ml which is comparable to the standard drug diclofenac sodium (100 mg/ml). The ethyl acetate extracts of *Skimmia anquetilia* leaves have anti-arthritic activity using *in vitro* methods such as human red blood cell membrane stabilization and protein denaturation [15].

Alangium salvifolium

Alangium salvifolium belongs to the *Cornaceae* family, is a flowering plant. It is mostly found in India's arid areas, such as plains and low hills, as well as along highways. Scientistsused a Carrageenan-induced oedema and cotton pellet-induced granuloma model to examine *in vivo*antiarthritic activity. F6 and F7 isolated from *Alangium salvifolium* leaves extract exhibited mild to strong anti-arthritic properties, due to the presence of polyphenol and flavonoids, according to the findings [16].

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Berberis orthobotrys

The *Berberis (Berberidaceae)* family, which includes around 500 species, is known in Persian as Zereshk. *Berberis orthobotrys* belongs to the *Berberis* genus and is a fragrant annual shrub.The main phytoconstituents are aporphine-benzylisoquinolines, chitraline, kalashine, khyberin, berbamine, and oxyacathine. Akber Dad [17]discovered three compounds in methanol extract of the entire plant -sitosterol, Eicosanol, and Sesamin.*A*nti-arthritic activity in Bovine Serum Albumin and Egg Albumin denaturation methods along with Complete Ferund's Adjuvant models were done by various scientists [18].The findings revealed that albumin denaturation was inhibited at various concentrations, as well as RBC membrane stabilization, with the best results obtained at 800µg/ml. Arthritic lesions and changes in body weight were better protected in CFA models. The aporphine-benzylisoquinoline alkaloids (berberine, berbamine), phenols and flavonoids presence detected in *B. orthobotrys* are the potent compounds involved in the anti-Arthritic activity.

Cissus quadrangularis

Cissus quadrangularis, also known as Asthisamhari, is a tropical and subtropical xeric wood plant in the *Vitaceae* family. In India's hotter regions, it can be found growing alongside hedges and in neighboring countries. The major bioactive compounds of *Cissus quadrangularis* are β-amyrin, α-amyrin, β-sitosterol, quercetin, friedlin, genistein, and daidzein[19]. *Cissus quadrangularis* has strong anti-arthritic properties when protein and albumin denaturation inhibition methods were used, which might be due to the presence of active polyphenolic contents, triterpenoids, flavonoids and alkaloids [20].

Climatis orientalis

Climatis orientalis belongs to the *Ranunculaceae* family of buttercups and is a deciduous vine or scrambling shrub native to Asia and Central Europe. Umme Habiba Hasan and Alamgeer used heat-induced protein alterations and HRBC membrane stabilization methods, as well as formaldehyde-induced arthritis, to investigate anti-arthritic efficacy in rats. Aqueous ethanol extracts inhibited protein denaturation and RBC membrane stabilization in a concentrationdependent and significant way between 50 and 6400 µg/ml. In formaldehyde-induced arthritis, it showed a significant reduction in paw swelling and diameter at doses of 50, 100, and 200 mg/kg, with the greatest effect at the highest dose. *Climatis orientalis* has anti-arthritic properties, which explains why it is used in folklore [21].

Dissotis tholloni

Dissotis tholloni (Melastomataceae) is a tropical plant that has long been used to treat and ease several ailments in traditional medicine. The major phytoconstituents are triterpenes, sterols, tannins, flavonoids, anthocyanins, saponins, and anthraquinones.Stephanie Flore Djuichou Nguemnangand others investigated anti-arthritic and anti-inflammatory function. The aqueous and methanolic extracts inhibit cyclo-oxygenase, 5-lipooxygenase and protein denaturation which shows that these two extracts can inhibit the production of prostaglandins and leukotrienes. At a dosage of 500mg/kg, aqueous and ethanolic extracts greatly minimize zymosan A-induced edema and in CFA induced polyarthritis rat models. As a result, *Dissotis tholloni* can be used to treat arthritis [22].

Momordica charantia

Momordica charantia (Cucurbitaceae) is a plant that is commonly cultivated for its edible fruit in Asia, Africa, and the Caribbean, and has been used for a variety of ethnic medicinal purposes. Alkaloids, glycosides, terpenoids, tannins, saponins and flavonoids, sterols, and mucilage were found in ethanolic and aqueous extracts of *Momordica charantia* fruits [23, 24]. To test anti-arthritic activity, researchers used formaldehyde, Ferund's adjuvant induced arthritis in rats, and a Collagen-induced arthritis model in mice. *Momordica charantia* could block cyclooxygenase-2 pathway during the progression of inflammation. According to the findings, the aqueous extract of *Momordica charantia* controls inflammation in adjuvant induced-arthritic and collagen-induced arthritis models in rats and mice at doses of 200 and 400 mg/kg, suggesting that it may have anti-arthritic efficacy [25].

Nauclea pobeguinii

The local population usually uses *Nauclea pobeguinii* in conventional treatments.Tsafack Eric Gonzaand his coworkersused a polyarthritis model induced in rats by Complete Ferunds Adjuvant. Phytochemical investigations of both methanolic and aqueous extracts revealed the presence of flavonoids, tannins, alkaloids, saponins, steroids and terpenoids. Both methanolic and aqueous extracts has been shown to reduce joint inflammation, hyperalgesia, improve hematological parameters, lower liver enzymes, regulate oxidative stress parameters, and restore inflammatory joint inflammation [26].

Pergularia daemia

Pergularia daemia, or trellis vine, is a hispid perennial vine in the *Apocynaceae* family that grows widely in the old-world tropics and subtropics. The leaves, stems, shoots, roots, and fruits have been used to extract chemicals like terpenoids, flavonoids, sterols, and cardenolide.Iffath Hina M and Caroline Rose concluded that ethanolic extract of this plant can be used to treat arthritis by investigating protein and albumin denaturation methods. The ethanolic extract of roots and leaves of *Pergularia daemia* was capable in controlling the production of autoantigen and inhibits protein denaturation in Rheumatoid arthritis. The ethanolicextract of roots produces increased percentage of inhibition of denaturation of albumin when compared to the leaves of *Pergularia daemia* [27].

Schisandra grandiflora

The *Schisandra grandiflora* plant, which belongs to the *Schisandraceae* family, has a wide range of ethnic medicinal properties. The phytoconstituents present in the aqueous extract of *Schisandra grandiflora* are terpenoids, flavonoids, alkaloids, and glycosides. Challa Pradeep Kumar and his coworkers used formaldehyde to cause arthritis in rats. Biochemical parameters which include paw volume measurement, acute toxicity test and serum CRP levels and radiological analysis were used to determine anti-arthritic activity in rats. The main aim of the study is to reduce swelling and inflammation in the joints without any side effects. *Schisandra grandiflora* aqueous extract has potent anti-arthritic activity against rheumatoid arthritis, according to the findings of this report [28].

Conclusion:

Review in this area has explored the potential applications on plants for their anti-arthritic activity due to the increasing side effects and expensive medication of synthetic drugs which has tilted the focus of researches on herbal medicines. This impeccably strengthens the data regarding plants and it may aidin advancementof research in the field of phytopharmacology. Therefore in this review an effort has been made to provide information about some of the medicinal plants that possesses anti-arthritic activity.

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PHARMACEUTICAL/MEDICINAL CHEMISTRY

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Drug discovery, in simple term,is identifying the lead compounds of various pharmacological activities which can be incorporated into the development process to obtain different drug substances. Hence, the entire process is tedious involved with so many fields such as biotechnology, biopharmaceutics, toxicology and pharmaceutical engineering. Medicinal chemistry is one of those fields, an interdisciplinary matter,aiming to help in the early phase of drug discovery by synthesizingmolecules with therapeutic or biological relevance and improve those already having it.It provides broad scopes in exploring hundreds of moleculesof different pharmacological activitywith greater diversity and flexibility in the field which subsequently allow the researchers to explore further with much confidence.

Now, drug molecules or those, which may have positive biological impact on the human body, come from different natural sources but nature provides most of them in numerous types of complexityin huge varietiesbut in little quantities. And it becomes very challenging to recognize these complex patterns so, the first initiative was taken by the scientists to explore all these types of molecules and study them from all aspects such as structural, functional, physical and chemical features etc.When, the collected data seemed sufficientto design them synthetically, scientists imitated few of them in laboratories while maintaining the same features, and, they have been able to design several analogues series, afterthat, next approach was to put these molecules under therapeutic assessment to determine if same results are obtained as in case of naturally occurring molecules.

Now the process of imitating these molecules are extremely difficult job to do as the complexity found in them is hard to achieve in exact fashion synthetically but newer types of chemistry such as click chemistry helps us to reduce difficulty to some extent. After the successful therapeutic assessment, next objective is synthesizing these molecules, which are considered to be safe and effective in human body, in large scale to undergo in the development process via formulation of the drug molecules.However, the entire drug discovery process is still in progress as there are lots of works to do to understand the science behind it, because it does not have any set of principals or laws under which scientists would work without being worried or gambling, but, instead of, it has been devising its own set of hypotheses over the years based

on the observations from different experiments. Medicinal chemistry has been integral part of early phase of discovery of new chemical entities, proving to become an art of designing drug molecules and science of rationalizing those designs providingall possible details.

It has two approaches such as classical approach and rational approach. In classical approach, scientists used to design molecules with particular activities but would end up designing other molecules with different activities which was due to lack of technology. Apart from that, discovery of the drug used to be carried out mainly by metabolism study and clinical observations. But, in modern approach, rational based drug design is widely accepted and it also has two parts structure based drug design andrational based drug design. Both of them is established on the application of extensive knowledge about biological target. In target based drug design, where the target can be enzyme, protein, receptor, or amino acids, there are many methods available such as Insilico methods; ab initio methods and de novo syntheses etc. which, of course, are based on computer aided drug design. All these modern methodologies such as molecular modelling and molecular docking work iteratively to provide closer to the exact knowledge about all these individualbiomolecular targets and their association with small drug molecules regarding their stability, reactivity, rigidity, and their mechanismsagainst the pathological condition in animal body.

In recent times, medicinal chemistry is developed in association with AI based tools such as schrodingers and pymol3D which further increase the importance in the modern day drug discovery. Recently, it is also helping in designing novel drug delivery systems and putting its impact on the field ofnanomedicines. Eventually, the drug discovery and development process is gaining some speed which is fruitful for researchers to work on them deeply and design some impactful molecules for human body.

Medicinal chemistry in combination with artificial intelligence will bring more sophistication and delicacy to answer to those complexities seen in the nature in the future. AI algorithm based operations are ultimate answer to find closer than before to the appropriate answers. Eventually, moderate to severe pathological conditions in human body would be solved and explained effortlessly.

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PHARMACOVIGILANCE: NEED, OVERVIEW AND CHALLENGES AHEAD

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Abstract:

Pharmacovigilance (PV) is a branch of pharmaceutical sciences which deals with detection, understanding, analysis/assessment and prevention of any adverse drug reaction and adverse event. Pharmacovigilance (also known as post marketing surveillance) is 4th phase of clinical trials of drugs. It is one of the obligatory activities which is undertaken by Marketing Authorization Holder (MAH) or its representative. While filing New Drug Application (NDA), a prospective Marketing Authorization Holder presents a limited clinical profile of the drug. This implies that safety profile of drug is not well established in broad population. Once Marketing Authorization Holder gets an approval, the drug (and the drug product) is used on millions of patients globally. Due to idiosyncrasy; population, globally, shows augmented as well as bizarre types of adverse reaction which is an immediate threat to safety profile of drug (and drug product). While doing post marketing surveillance, Marketing Authorization Holder collects information in relation to any adverse drug reaction and adverse event that happens during clinical use of drug. Such collection of data on safety profile can be passive or active. Individual reports collected in such pursuits by Marketing Authorization Holder are called Individual Case Safety Report (ICSR). A large number of adverse drug reactions (serious & non serious) and adverse events reported constitutes signal which may warrant either dose modification or in some cases withdrawing of drug product. It is worth to note that some countries have well developed pharmacovigilance system while others have poorly developed or no system at all which is ultimate challenge in establishing the safety profile of the drugs. In some countries where doctor to patient ratio is very low; adverse drug reaction reporting is very feeble. There is an immediate need that all the members of health care team (doctors, registered nurses and pharmacists) must actively participate in adverse drug reaction reporting. Also, pharmacovigilance system must be introduced amongst public. They must be told about the modalities (like phone call, social media platforms and e-mail service) by which such reactions

can be reported. Only then benefit to risk ratio analysis can be properly done and safety profile of the drug can be checked and established fairly.

Keywords: Pharmacovigilance, Post Marketing Surveillance, Marketing Authorization Holder, Individual Case Safety Report, Social media

Background:

Appropriate dose is something which differentiates a drug or medicinal agent from poison[1]. Drug or medicinal agent is something which can be used for the purpose of diagnosis, mitigation or treatment of ailment or a disorder in humans or animals [2].A medicinal agent often tends to behave differently in different person or contrary to it; body of patient/recipient may respond differently to the different drugs; a phenomenon called idiosyncrasy [3]. This warrants that a drug's safety profile must be recorded even after it has been approved and marketed and hence government made it necessary for the companies to record the incidences of adverse drug reaction (ADR) giving rise to a field called pharmacovigilance [4-6].

Pharmacovigilance (PV) is 4^{th} phase of clinical trial where post marketing surveillance of drug/medicinal agent is done [7].Pharmacovigilance aims on early detection, understanding, early assessment and prevention of ADR that too with high fidelity. Pharmacovigilance program is well established in developed countries like USA and Europe but in developing countries like India; the Pharmacovigilance program is in developing stage. ADRs are often under-reported on account of negligence of stake holders of drug safety.There is a need that healthcare professionals and/or patient may report ADRs conveniently so that this system flourishes and an appropriate safety profile of drug becomes visible to all [8-13].

Initiation of pharmacovigilance activity: aftermath of thalidomide incidence:

First scientifically plausible ADR known to human is thalidomide incidence of 1961. Thalidomide was manufactured and marketed in 1950s as anti-flu, soporific and later as a drug to prevent morning sickness during pregnancy. Recipients were unaware of teratogenic effect of thalidomide since its safety profile was largely unknown. Thalidomide caused phocomelia (seal babies). According to a report; around 10,000 children were born with disfigurement of lower limbs [14-17].

Accounting on this; in 1968, 10 countries of world (Canada, The Netherlands, Australia, USA, Sweden, Czechoslovakia, Ireland, Germany, New Zealand and UK) along with their health agencies collaborated with WHO (World Health Organization) and it was the date when trial run for international drug monitoring was conceived.

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Present scenario of international drug monitoring:

Currently international drug monitoring is taken care of by Uppsala Monitoring Centre (UMC) of World Health Organisation located in Sweden. This is an independent centre dedicated to scientific research and drug safety with aim of providing safe and effective medicines to patient. UMC also maintains a global drug safety database known as vigibase. All the health agencies of world (CDSCO, USFDA and EMA to name a few) collaborates with UMC and share their safety data also. UMC intends to prevent unnecessary harm to the patient by maintaining not only global safety database but also by providing pharmacovigilance trainings and by issuing global guidelines on drug safety [18-21].

Adverse drug reaction, adverse drug event and criteria for seriousness of case:

Adverse drug reaction can be defined as unintended and unpleasant response that happens during the course of treatment with drug at clinical doses. Adverse Drug Reaction has definite clinical attribution/causal relationship with the drug and its therapeutic dose [22-25].

Adverse Drug Event is an untoward medical condition caused during treatment with drug. Adverse Event has no clinical attribution/causal relationship with the drug and its dose.

Causal relationship is something which distinguishes an ADR from ADE. This can be understood with the help of example. Sedatives may cause loss of consciousness at high dose (a side effect) but during this course if the patient fell down and gets a fracture then fracture is an adverse event (fracture has no causal relationship with sedative and its dose)[26, 27]

An ADR may be called serious if it meets any of the given criteria [28-37]:

1. Causes hospitalization or is immediately life threatening.
2. Prolongs existing hospitalization
3. Leads to death.
4. Causes disability or incapacitation
5. Is clinically significant

Case making in pharmacovigilance:

ADR monitoring is an obligatory activity (mandated by health agency of a particular country) which has to be undertaken by the company which has been awarded authorization for marketing a drug.

The company either by herself or by hiring services of third-party vendors records the ADRs and make cases. For a case to stand valid; there should be 4 criteria that should be met [38, 39]:

1. A distinguishable patient.

2. A suspected drug.
3. An ADR/Adverse Event (AE).
4. A perceptible reporter.

In the absence of any of the aforementioned criterion; the case stands invalid and is not recorded by the company [40].

What after case has been made?

A single case made in pharmacovigilance process is called as Individual Case Safety Report (ICSR).The marketing authorization holder has to submit reports (on a periodic basis) to the health agency/drug regulator of the territory. Two reports are made: Periodic adverse drug experience reports (PADERs) and periodic safety update reports (PSUR)/periodic benefit-risk evaluation reports (PBRERs).

If PSUR contains large number of serious cases then a signal is generated. It is worth to note here that signal contains information either on ADR data suggesting new safety information or on changed risk to benefit ratio. Signal so generated is validated, confirmed, analyzed, prioritized and assessed. MAH or marketing authorization holder is then suggested recommendations for action by regulatory authority of that country [41-44].

If such signals continue to exist or if there is a significant change in the safety profile of the drug then marketing authorization of the drug is cancelled and drug product is recalled from market.

Table 1: Timelines for reporting ICSR (USFDA timelines) [45]

Type of case	Timeline
Suspected unexpected serious adverse reaction (SUSAR)	7 calendar days
Serious adverse reaction (SAR)	7 calendar days
Clinical trial other Serious cases	15 calendar days
Non serious Cases	30 calendar days

Here day zero (0) starts on the day when MAH has received all the pertinent information to make case.

Table 2: Timelines for reporting Periodic reports (USFDA timelines) [45]

Time-period till approval	Periodicity
For 3 years after approval	Quarterly periodic report
After 3 years	Annual reports

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Pharmacovigilance scenario in India:

In India, PV activities in undertaken by National Co-ordination Center (NCC), Ghaziabad (U.P). This entire program flourishes under Ministry of health and family welfares, Government of India. N.C.C receives reports from zonal ADR monitoring centers (they in turn are reported by regional and peripheral ADR monitoring centers). NCC is supported by CDSCO & its zonal offices. NCC, CDSCO & Ministry of health and family welfares, Government of India together take decision on continuing and banning of drugs based on signals [46-52].

Although India has a fair PV structure still the number of cases is under reported because of three reasons:Less number of physicians available for number of patients (The doctor-population ratio in India is 1:1456 against the WHO recommendation of 1:1000 in 2020). Physicians are often busy with treatment and find no time for reporting ADRs.Lack of awareness in other health care team members (nurses and pharmacists) regarding their role in PV process.Lack of awareness in patients regarding PV process. They hardly know that each ADR reporting is contributing to global heath.

The need of the hour is that all stakeholders should proactively participate in PV process.

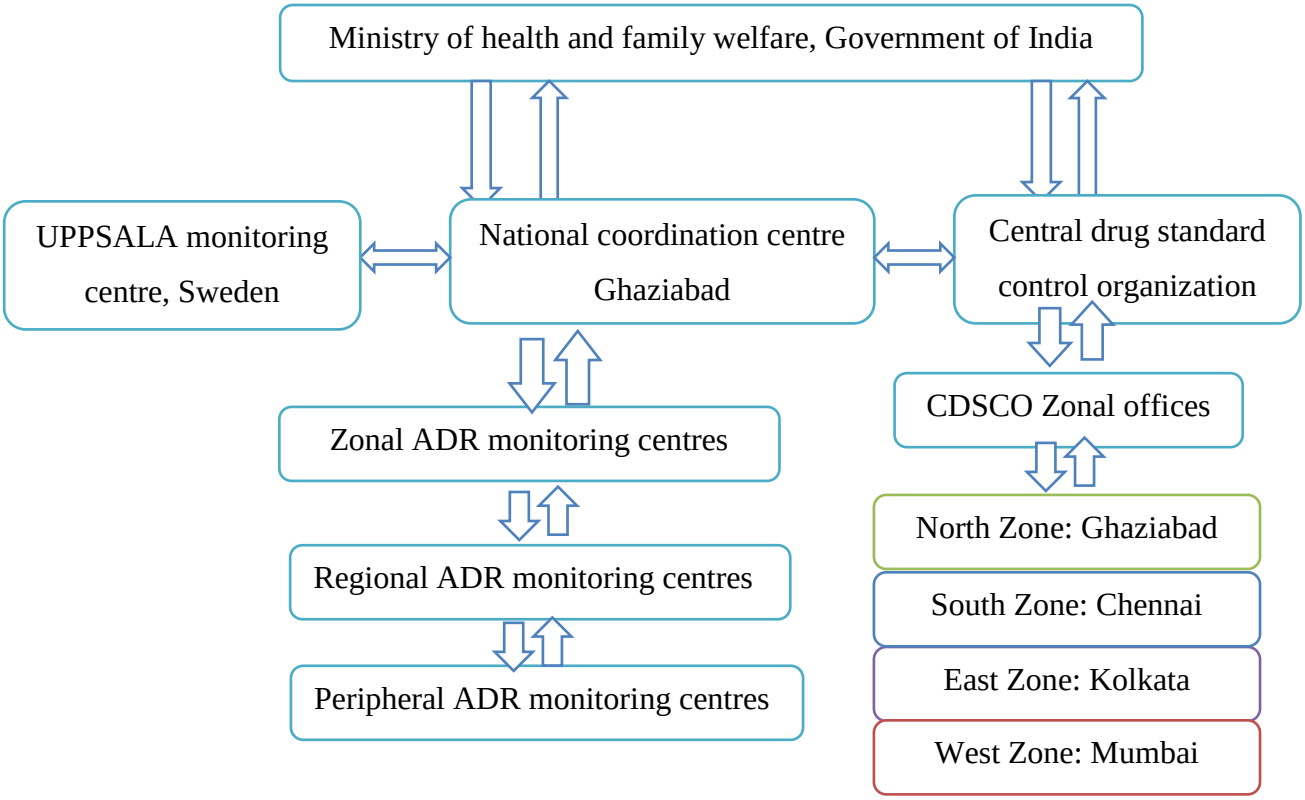

Figure 1: Pharmacovigilance program in India

Conclusion:

Pharmacovigilance is underdeveloped field in some developing countries which is one of the biggest challenges to the safety profile of drug.

It must be understood that the safety and efficacy of the drug is checked on the limited people (approximately 5000 people) before marketing authorization is granted and when drug is used in a large population, there can be a significant change in the benefit to risk ratio of drug which may warrant dose modification or even withdrawing of the drug.

Hence government of distinguished countries should take expedient steps in order to apprise public as well as health care professionals to participate in the pharmacovigilance process and contribute to safety profile of the drug. ADR reporting must be made such convenient that a consumer/patient/medical practitioner can report to company even by using social media platforms [53-67]. After all it is rightly said that due diligence is better than regret.

Abbreviations:

ADE: Adverse Drug Event, CDSCO: Central Drug Standard Control Organization, EMA: European Medicines Agency, ICSR: Individual Case Safety Report, MAH: Marketing Authorization Holder, NCC: National Co-ordination Centre, PBRER: Periodic Benefit-Risk Evaluation Report, PMS: Post Marketing Surveillance, PSUR: Periodic Safety Updates Report, PV: Pharmacovigilance, UMC: Uppsala Monitoring Centre, USFDA: United States Food & Drug Administration, WHO: World Health Organization.

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EFFECTIVE PHARMACEUTICAL DRUG TREATMENT AGAINST SARS-CoV-2 INFECTION

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Abstract:

Corona virus disease 2019 (Covid-19) is one of the most severe pandemics declared as Public Health emergency of international concern by World Health Organization on 20th January 2020, responsible for millions of deaths and still peoples are suffering symptomatically and asymptomatically along with number of secondary infections. As priority in the therapeutic management various structures of drugs, their mechanism of action (Inhibition of viral RNA Polymerase enzyme, endocytic pathway, sialic acid receptor mechanism and antiviral effect) in relation with the location of virus and their destruction, applications and side effects were studied and found Remdesivir, Chloroquine, Hydroxychloroquine and Favipiravirare effective drugs for the treatment of SARS CoV-2 virus. Also, convalescent plasma therapy which is classic adaptive immuno-therapy largely applied for the prevention and treatment of Covid-19.

Keywords: SARS CoV-2, ACE2, Remdesivir, Plasma therapy, S Protein, Immunomodulators

Introduction:

From the last manycenturies deadliest pandemic had taken place which causes death of the many people in the world. Some of them are Justinian plague (541-542 B.C.) caused due to *Yersinia pestis*-nearly about 40 million deaths i.e. 19.1% of the population had loss their life in this pandemic. Highly contagious Smallpoxdisease caused due to Variola virus in which skin eruption like pustules form and mortality rate was 30% and Edward Jenner had developed the world first vaccine in the 1798. In the first decade of last century in the year 1918-1920, Spanish flu occurred in the setting of modern medicine and at this time the H1N1 strain of the Influenza virus had spread to every corner of the world around 45 million of individual lost their life. After this Spanish flu - SARS (Severe Acute Respiratory Syndrome) was the first outbreak in the Twenty-first century in the year 2002-2003 and approximately 770 people loss their life in this outbreak. Then, first case of Ebola virus (2013) found in the central and west Africa and

outbreak in the remote in Guniea in the December 2013. There were about 28,000 cases and the 11,000 fatalities. After that Zika virus, a little dormant virus found in the rhesus monkey in the Uganda in 2014 only outbreak among human recorded in Micronesia in 2007 and virus was identified in the Brazil in 2015 (Damir, 2019).

Currently, Corona virus disease 2019 (Covid-19) outbreak was in Wuhan City, Hubei Province, China in the month of December 2020, caused by a novel severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) and on 30th January 2020, World Health Organization (WHO) declared the outbreak as Public Health Emergency of International Concern (Harapan et. al Covid-19 virus is highly infectious and caused high mortality across the world led to 3.5 million deaths and may more death in near future, if proper therapeutic treatment not given. There are many variants of concern and Variant of interest for giving correct treatment for covid-19 disease. As on 8th June 2021, there have been 173,271,769 confirmed cases of COVID-19, including 3,733,980 deaths, reported to WHO and total of 1,900,955,505 vaccine doses have been administered till 5th June 2021 (https://covid19.who.int/).

Coronaviruses are large, enveloped, positive stranded RNA viruses responsible for infecting a wide variety of mammalian and avian species. If you see the phylogenetic relationship in the Coronavirinae subfamily which is formed by mainly four genera as Alphacoronavirus, Betacoronavirus (four lineages A, B, C and D), Gammacoronavirus and Deltacoronavirus. Corona viruses are non-segmented enveloped virus with single stranded RNA ranging about 26-32 Kb in length. The spherical shape of the corona virus and the size of the virus is about 60-140 nm and the outer surface of the studded with the distinctive 9-10 nm long (Anushmali et. al, 2020). The novel corona virus has four structural proteins. One of them called the spike proteins, mediates the virus entry into a host cell by binding to an enzyme attached to the cell called angiotensin converting enzyme2 (ACE2) (Squarene *et al.,* 2020).Once the virus is infected to the body it attacks the respiratory system then the virus releases its RNA and uses the RdRp complex to replicate its genome.

Symptoms of Covid-19:

The people with Covid-19 had wide range of symptoms from mild symptoms to severe illness, may appear 2-14 days after exposure to the Virus. The symptoms include Fever or chills, Cough, Shortness of breath or difficulty breathing, Fatigue, Muscle or body aches, Headache, New loss of taste or smell, Sore throat, Congestion or runny nose, Nausea or vomiting, Diarrhea, Conjunctivitis, Loss of taste or smell, Rash on skin, or discoloration of fingers or toes (www.cdc.gov/).

Drug Treatment for Covid-19 disease:

The current therapeutic management for covid-19 mainly includes antiviral drugstherapy (Remdesivir, Hydro-chloroquine, Ivermectin and Tocilizumab) Plasma therapy, Immunomodulator agent (corticosteroids, Interferon β-la, Interleukin(IL-1) and Anti-IL-6 receptor monoclonal Antibodies)are used for treatment of covid-19 patients (Cascella *et al.*, 2020). SARS-CoV-2, is a single-stranded RNA-enveloped virus, targets cells through the viral structural spike (S) protein that binds to the angiotensin-converting enzyme 2 (ACE2) receptor. After receptor binding to enter inside the cell, virus particle uses receptors of host cell and endosomes to enter inside cells. A host type 2 transmembrane serine protease, TMPRSS2, provide facility to enter intocell via the S protein (Hoffman *et al.,* 2020). After that synthesis of viral polyproteins takes place which encode for replicase-transcriptase complex. Then virus synthesizes RNA using its RNA-dependent RNA polymerase enzyme.

Structural proteins are synthesized leading to completion of assembly and release of viral particles (Chen *et al.,* 2020; Fehr and Perlman 2015; Fung and Liu, 2014). These viral lifecycle steps provide potential targets for drug therapy. Promising drug targets include nonstructural proteins (e. g, 3-chymotrypsin-like protease, papainlike protease, RNA-dependent RNA polymerase), who share similarity with other novel coronaviruses (nCoVs). The mechanism of action of drugs and major pharmacologic parameters of treatments or adjunctive therapies for COVID-19 is given below (James *et al.*, 2020).

Remdesivir:

Remdesivir is the prolong drug which intracellularly metabolizesand structurally analogue to adenosine triphosphate which inhibit the viral RNA polymerase. The main structural feature distinguishes remdesivir form the adenosine is the modification of specific chemical molecule. This drug has the broad-spectrum activity against Filovirus and Corona virus family members. In-vitro testing observed that remdesivir has the activity against the corona virus (Saqrane *et al.*, 2020).

Structure of Remdesivir:

It is essentially a modified version of the natural component of adenosine which is essential for formation of DNA and RNA. The active form of the drug has the three-phosphate group recognized by the viral RNA polymerase enzyme. When the remdesivir come in the contact with the virus and the virus mistakenly incorporates it into the copies of the RNA instead of the adenosine triphosphate. The compound also evades the proofreading enzyme by slotting itself into the RNA at the slightly different position from where the adenosine triphosphate is supposed to be. Once Remdesivir is incorporated into the RNA growth chain, the presence of

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carbon and nitrogen group can cause the sugar to bend which in turn distorts the shape of the RNA chain, therefore only three additional nucleotides can be added. By adding these nucleotides, it stops the synthesis of RNA strand and eventually interrupts the virus replication mechanism. As the corona virus has specialized mechanism which recognizes the artificial nucleotides and eliminate the artificial nucleoside and prevent the replication of the corona virus (Eastman *et al.,* 2020). Ultimately the RNA synthesis process has be stop. Recently, the U.S. national institute of Allergy and infection disease conducted clinical trials with Remdesivir and found it is effective for the covid-19 virus. U.S. Food and Drug administration granted emergency approval for the use of the remdesivir as an effective antiviral drug against Covid-19 (Clavin *et al.,* 2020).

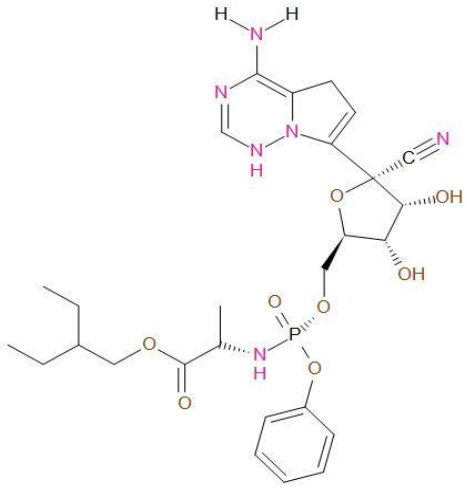


Figure 1: Structure of Remdesivir

Application of the Remdesivir:

It is anintravenous nucleotide drug which contain Adenosine analog which bind to the viral RNA dependent RNA polymerase and inhibit the viral replication through premature termination of RNA activity against SARS-CoV-2 virus.

Side effects of the Remdesivir drug:

1. An infusion reaction may occur with remdesivir with symptoms such as the low blood pressure, nausea, vomiting, sweating and shivering.

2. In the case of the serious allergic reaction symptoms such as the rashes, itching, swelling, severe dizziness.

Chloroquine (CQ) and Hydroxy-Chloroquine (HCQ):

Hydroxy-chloroquine is the safer analogue of the chloroquine which is used as antimalarial and immuno-modulator against the covid-19. It hasantiviral activity in which decrease in the phago-lysosome fusion, increasing intracellular pH and imparting viral receptor glycosylation. Immune-modulatory effect responsible for decreasing production of cytokines especially IL-1 and IL-6 and inhibiting the toll-like receptor (Arshad *et al.,* 2020).

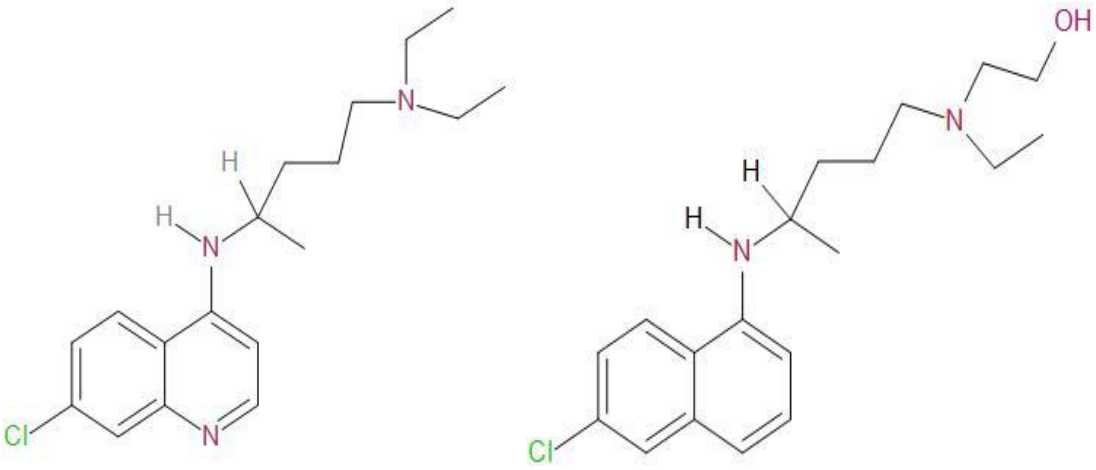


Figure 2: Structure of chloroquine andhydroxy-cholorquine:

Some of the researcher proved that hydroxy derivative is safer than the CQdue to the decrease in the renal and ocular toxicity,so it is used as a substitute for CQ. There are some following ways thatHQC and QC showed some pathway to inhibit the covid -19 virus.

Mechanism of action of CQ and HCQ by the endocytic pathway:

As Covid-19 virus enter into the host cell by endocytic pathways. Pneumocytes are CtBP-1 & 2 and pak proteins are important for the micropinocytosis which is an essential mediator in the Pneumocytes endocytic pathway. As chloroquine get collected in the endosomes and lysosomes,it leads to the pH neutralization and little hindrance in the effect of the proteases, which preventing the S protein cleavage (Satarkar *et al.,* 2020). Chloroquine inhibits the fusion of the lysosomes due todysregulation of Sytaxin which hamper the golgi functioning and block transportation essential material into lysosomes.

Hydroxy-cholorquine prevents the movement of SARS-CoV-2 virus, form the early endosomes to early lysosomes that are important for the viral release in the host. Increases in the pH of the lysosomes and endosomes are mediated by hydroxyl-cholorquine further lead to autophagosomes which break the S protein which prevents the membrane fusion.

Sialic acid receptorinhibitory mechanism:

Recently, it has been found that MERS-CoV virus binding occur at the receptor of the sialic acidand it identified at the N-terminal of the Sprotein in the SARS-CoV-2. This mediates the entry of the sialic acid receptor in the upper respiratory pathway and this receptor was previously known as ACE-2 receptor. There is another binding site for binding of gangliosides in the SARS-CoV-2, S proteins N- terminal domain (NTD). Especially, 9-O-SIA variant inhibiting the sialic acids by the drug Hydroxy-chloroquine and chloro-quine. However, study proved that hydroxy-chloroquineshows better potency than chloroquine.Epithelial cells of the conjunctiva and cornea has the 2-6 linkage sialic acid receptors as the SARS-CoV-2 is also speculated to enterthrough the binding with alpha 2, 6 linkage and alpha 2, 3 linkages of the sialic acid receptors. Nasolacrimal regionpossess both the receptor therefore respiratory passage via duct of nasolacrimal lead to successful entry into the host cells. (Satarkar *et al.*, 2020).

Application of Hydroxy-chloroquine:-

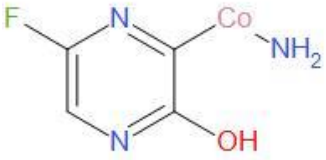
1. This drug decreases pH of the lysosomes and endosomes.
2. used in a treatment or prevention of Malaria.
3. Also employed in the treatment of lupus.
4. It is used in arthritis to stop swelling and pain.

Side effects of Chloroquine and Hydroxy-chloroquine:

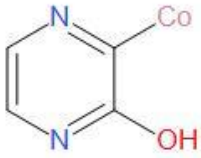
1. A higher dose of the hydroxyl-chloroquine cause the irreversible retina damage which may cause the risk of retinal damage higher in people with the pre-existing eye problem, kidney disease.
2. There are many symptoms such as vision change or damage to your retina, heart disease, heart rhythm disorder, diabetes, a stomach disorder, an allergy to quine, liver or kidney disease, porphyria (a genetic enzyme disorder that cause symptoms affecting the skin or nervous system), headache and Nausea (Alanagreh *et al.*, 2020).

Favipiravir:

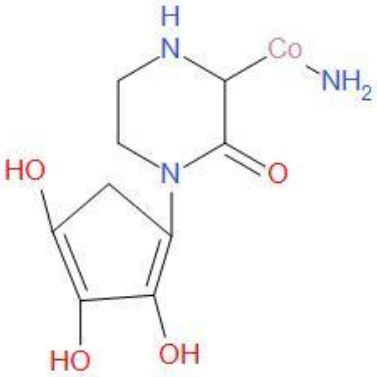
The chemical name of the Favipiravir is 6-Fluoro-3-oxo-3,4-dihydropyrazine-2-carboxamide alternatively, it is also called as T-705, Fapilavir and Favilavir. The class of this antiviral agent. Flavipiravir is derived by chemical modification of the pyrazine moiety of T-1105.



Favipirain (T-705)



T-1105



T-1106

Favipiravir drug is administered as a prodrug which has low distribution of volume and 54% of the protein binding. This drug has a short life cycle about 2.5 to 5 hours leading the rapid renal elimination in hydroxylated form. This elimination is maintained by the enzyme aldehyde oxidase and marginally by Xanthine oxidase. This drug is exhibit in the two form that it is dose dependent and time dependent pharmacokinetics. This drug is not metabolized by the P-450 system but inhibit the component i.e CYP2C8 hence, it is necessary to maintain the precaution while co-administration with this drug. (Agrawal et.al 2020).Some studies shows that there is binding of the F-RTP to the active site of the SARS-CoV and MERs-CoV in the presence of the agent of protein as the F-RTP bound to RNA terminal in the presence of the magnesium ions, nucleotide phosphate and RdRp proteins (NSP-12, NSP-7and NSP-8). This drug bind to the protein of the SARS-CoV-2 to the NPS-15 protein which is endonuclease of the SARS-CoV-2 plays an important role in the replication of the virus RNA. This distinct mechanism underlying of favipiravir-mediated interaction with corona virus RdRp.

Mechanism of the Favipiravir:

When the covid-19 virus enters into the host cell, tissue with the molecule undergoes phosphor-ribosylation to Favipiravir-RTP which is the active form of this drug which shows the antiviral drug effect on the SARS-CoV-2 virus. This molecule act as the substrate for the RNA-dependent RNA-polymerase (RdRp) enzyme which not taken the purine nucleotide in the SARS corona virus thus inhibiting activity of this enzyme lead to the termination of the viral protein synthesis. As this enzyme get incorporated with the viral RNA strand which inhibit the preventing the further extension and stop the replication of the SARS-CoV-2. The mechanism of action along with preservation of the catalytic domain of the RdRp enzyme across the various virus show broad spectrum of this drug (Sada *et al.*, 2020).

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Application of Flavipiravir:

Purine nucleotide from the Favipirin drug inhibits the enzyme activity of which lead to the termination of the viral protein synthesis.

Side effects of the Flavipiravir:

Some Japanese studies observed that, there are acute reaction seen in the individual who are treated with these drugs they show relatively minor and include the Hyperuricemia and diarrhea in the 5% of the patient and there is reduction in Neutrophile count in 2% percent of the patients.

Side effects	**>1%**	**0.5-<1%**	**0.5%**
Hyper-sensitivity		Rash	Eczeme. pruitus
Gastro-intestinal	Diarrhea	Nausea, vomiting, adominal pain	Adominal discomfort dudodenal ulcer, heematochezia gastritis
Hopatic	AST(GOT) increased ALT (GPT) increased $_{\gamma}$ -GTP increased		Blood ALP increased blood hillirubin increased
Hemotologic	Neutrophil count decreased, white blood cell decreased	Glucose in the urine present	White blood cell count increased reticulocytes count decreases monocytes increased.
Metabolic disorder	Blood uric acid increasedBlood triglycerides increased		Blood potassium decreased.
Respiratory			Asthma, Oropharyngeal, pain,rhinitis, Nasopharygitis
Other			CPk increase blood urine present tonsil poly pigmentation, Dysgeusis, bruise.

Convalescent plasma therapy treatment:

Convalescent plasma therapy has been classic adaptive immuno-therapy applied to the prevention and treatment of Covid-19 and various kinds of diseases. As the pathogen enter into

the body of the individual there are two lines of defense i.e. innate immunity and the acquired immunity. The innate immunity contains number of the soluble cell-based antimicrobial factors, and they get triggered as soon as the infection takes place in the individual.Adaptive immunity consists of the pathogen-specific antibody and T-cells and develops later and contribute for fighting with pathogen and to eliminate the activity of the pathogen. In addition to the adaptive and innate immune system, some of the immuno-modulators, immuno-suppressants drug can also stimulate immune system and helps to remove the pathogen or suppress the activity of the pathogen (Rizk *et al.*, 2020) lives. So one dose of the 200 ml of the convalescent plasma can help an individual to fight for the covid-19 virus and eliminate it or suppress its effect. While clinical symptoms significantly improved with the increase in the oxygen level of an individual within approximately 3 days as we know that corona virus has one of the rapid viral replication system and ability for massive inflammatory cell infiltration and elevated pro-inflammatory cytokines or even cytokines storm in the alveoli of lungs results in the acute pulmonary injury and acute respiratory distress.

As the recent, studies found that in covid-19 that lymphocytes count in the peripheral blood were reduced and the level of the cytotoxin in the plasma requires the ICU support IL-6, IL-10. Recovered Covid-19 patients contain the large number of the humoral immunity against the virusand large neutralizing antibody which is highly capable for neutralizing the SARS-CoV-2 and remove the pathogen from the blood and from the pulmonary tissue too. There is decrease in the antibody of the SARS-CoV-2 decrease gradually after the 4 months after disease process. If the antibody tiler decreases rapidly within the 3monthshence, study suggest that the humoral immune response from the plasma of the recently recovered patient more is effective. The recently recovered Covid-19 patient has neutralizing antibody tiler above the 1:640 and hence they are the suitable for the plasma donation for saving the other patient life by plasma therapy (Duan *et al.*, 2020).

Conclusion:

From the above detail study of various therapeutic drugs and therapy it is clear that all the drugs are highly capable for the controlling the covid-19 virus However, when the covid-19 emerged in the world, these drugs have played a critical role of saving lots of lives.So,these drugs have proved as the life-saving drugs to some extent.

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DRUG AND PRO-DRUG DESIGN

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Abstract:

Pro-drug plays a very essential role in the field of pharmaceutical drug discovery and development, by improving the pharmacokinetic, biopharmaceutical property of the medicinally active agent and produce the desired therapeutic effect at the site of action. The term pro-drug is defined as the derivative of the active drug molecules which are biologically inert and produced the pharmacological effect by in-vivo conversion of the enzymatic and chemical. the main aim of the development of pro-drug in pharmaceutical drug discovery and development to overcome some incompatibility that is effective to produce the desired pharmacological effect that is chemical instability, low solubility, pre systematic toxicity, low target selectivity, etc. in this review we have discussed the concept, classification and some example of the pro-drug which are used to produce the desired pharmacological effect by binding to the targeted site.

Keywords: Pro-drug, pharmacokinetic, biopharmaceutical.

Introduction[(1)]:

The term pro-drug is defined as the derivative of the active drug molecules which are biologically inert and produced the pharmacological effect by in-vivo conversion of the enzymatic and chemical. Basically, it helps to hide the physicochemical and pharmacokinetic (ADME) barrier by improving the pharmacokinetics and produced an effective pharmacological effect.

History of pro-drug[(2)]:

The term pro-drug was introduced in the middle of the 20th century by Albert in 1958. The first pro-drug was introduced in the year 1867 is acetanilide which is the antipyretic agent by

Cahn and Hepp. After that phenacetin (acetophenetidin) is the analgesic drug that was introduced in the year 1897 and asprine in 1899.

Concept of pro-drug[3]:

The aim to developed and design the pro-drug in pharmaceutical drug discovery and development to inhibit or mask some incompatibility that is the effect the pharmacological activity are the low solubility, chemical instability, pre systematic toxicity, low target selectivity, etc. pro-drug are the derivative of the active drug and produce the desired effect after the administration to the body by the enzymatic and chemical reaction after chemical changes of the active drug. Pro-drug is designed to maintain the pharmacokinetic of the parent drug by optimizing the absorption, distribution, metabolism, excretion profile of the active drug.

Classification of the pro-drugs:[4]

Pro-drug is classified into two different groups are-

1. Carrier–linked pro-drugs
2. Bio precursor pro-drug

1. **Carrier–linked pro-drugs:**

In this, the inert carrier is linked with the active compound covalently and improved the lipophilicity of the drug. They consist of by attachment of the drug to the carrier group. And the active drug released by enzymatic or nonenzymatic mechanism. Carrier–linked pro-drug are further classified into:

- Double pro-drug or cascade- latentialed pro-drug: in this, the drug is released only by enzymatic conversion.
- Macromolecular pro-drug: in this, the macromolecules are used as a carrier to transfer the drug to the active site.
- Site-specific pro-drugs: transport the drug to target active site
- Mutual pro-drug: in this, the two pharmacologically active compounds are linked together in place of inert molecules to produced the effective pharmacological effect.

2. Bio precursor pro-drug:

This type of pro-drug is active in itself to change into the active drug after the metabolism and produced the effective pharmacological effect on the site of action.

Objectives of pro-drug[5]:-

- Most of the drug is produced the effective pharmacological effect and used to treat the many types of serious disease but the toxicity and the other parameter which does not come under

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the stander criteria are the reason for not using these drugs. After the pro-drug design, these types of drugs are used for the treatment of various types of disease by optimizing the physicochemical and pharmacokinetic parameters of a drug.

- Transfer the drug to the active site by encapsulating the drug in the carrier which is accepted by the barrier to enter the pro-drug and transfer the active drug to the respective site.
- Improve the kinetic of the drug.

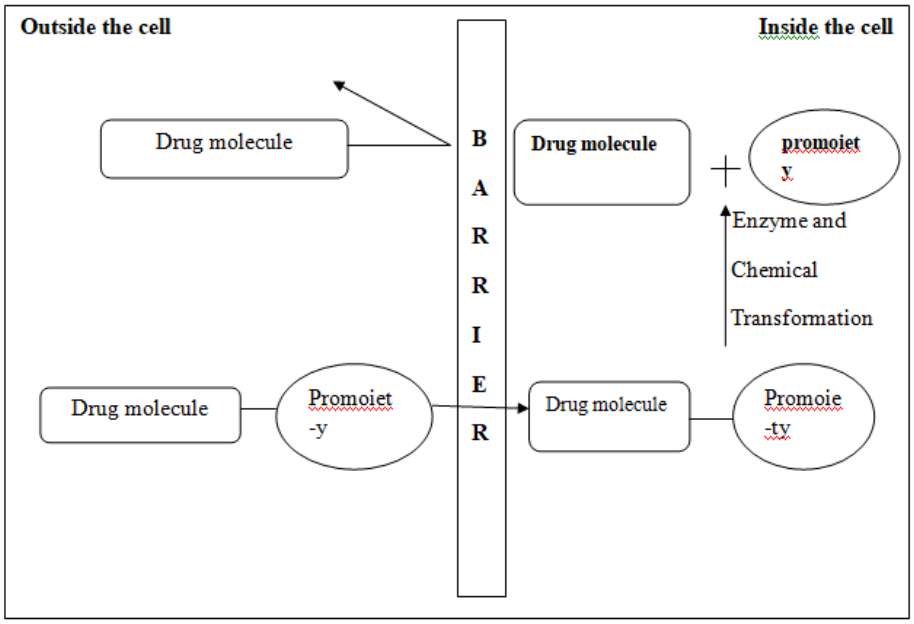

Figure 1: Concept of pro-drug

Pro-drug incorporated drug delivery system[(6)]:-

In this drug delivery system, the active compound is encapsulated and released in systemic circulation in a controlled rate land work like a controlled and sustained released drug delivery system.

Liposome[(7)]:

Liposomes are the drug delivery system in which the drug is incorporated either in the aqueous or lipid bilayer according to their physicochemical property and released the drug at pre defined rate.

Advantages of liposome:

- Drug Carrier for both aqueous and lipid medium.
- Controlled and sustained release drug delivery system
- Control hydration.

Disadvantages:

- Short half-life.
- Leakage of encapsulated drug.
- Less stable.

Lipoprotein [8]:

Lipoproteins come under the family of the macromolecular complexes. Lipoproteins are the carrier that transfers the drug (primarily cholesterol and triglyceride) to the active site.

Emulsion:

Emulsion helps to transport the lipophilic drug to the targeted site by making the oil in water emulsion.

Application of pro-drug:[9] Pro-drug are used to optimize both biopharmaceutical and pharmaceutical activity to modify the drug pharmacological activity.

- Change the taste- bad taste is caused the poor patient compliance. to overcome bad taste two approaches can be done are-
  - Solubility of the drug reduces in saliva.
  - Lower the drug affinity to taste receptors.
- Improved the odour of the drug which improved patient compliance.
- Change the physical form of the drug for formulation stability.
- Reduction in GIT irritation:- some drugs are cause irritation on GIT and to overcome this problem pro-drug are used and reduced the problem causes by the drug on GIT.
- Chemical stability is improved by using the pro-drug.
- Improved the drug bioavailability by improving the lipophilicity of the drug.
- Prevent the presystemic metabolism.
- Reduced the toxicity of the potent drug.
- Dileveard the drug to their active site.

Pro-drug and their uses:[10]

Sr. No.	Pro-drug	Therapeutic uses
1	valacyclovir	Herpesvirus
2	Tenofovir alafenamide	HIV/ AIDS and chronic hepatitis
3	selexipag	Pulmonary arterial hypertension
4	Baloxavir marboxil	influenza
5	prasugrel	Prevention of thrombotic and cardiovascular events.
6	remdesivir	Coronavirus disease 2019 (COVID-19) in adults and adolescents with pneumonia requiring supplemental oxygen.

Conclusion:

Pro-drug is designing is one of the most effective approaches in pharmaceutical drug designing and development. Most of the active drug which more effective and produced the Better pharmacology effect are not come under the criteria for designing the new active medicine for medical uses just because they do not pass some evaluation parameters, not compatible with the excipients of the formulation, biopharmaceutical parameters like (ADME) are not good and some other issues like the toxicity of the drug, etc. To overcome these problems pro-drug are play a very effective role in the development of a new entity or drug by hiding and optimizing the undesirable data and make the formulation of low toxicity and increased pharmacology activity. Pro-drug is helping to transfer the active drug to their site of action without any hindrance, hide the bad odour of the drug, and improved patient compliance.

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FORMULATION AND CHARACTERIZATION OF FASTER RELEASE FORMULATIONS OF NIMESULIDE BY SOLVENT DEPOSITION TECHNIQUE

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Abstract:

Faster Release formulations of Nimesulide by solvent deposition technique using carrier such as starch, Aerosil, and Microcrystalline cellulose in the ratio 1:2 and 1:4 was formulated and characterized Release of Nimesulide from all the formulations and the Release was in the following order **Starch < Aerosil < Microcrystalline Cellulose.** Dissolution rate of Nimesulide was increased as the concentration of the excipient was increased. Stability studies indicates that the formulation was stable. IR spectral studies reveals that there is no interaction between added drug and excipient. Invivo studies confirms the anti inflammatory activity of solvent deposited system of Nimesulide.

Introduction:

Recently fast dissolving drug delivery have started gaining popularity and acceptance as new drug delivery systems, because of their gain patient compliance, and it is easy to administer. Nimesulide, a category of Non steroidal anti-inflammatory drug, with a broad spectrum of activity was used in verity of inflammation and pain states and it is administered in the form of tablets and oral suspension in divided doses and it is also applied as Transdermalgel. Nimesulide is water insoluble drug[1,2] but absorption, Bio availability and pharmacological Response depends upon the aqueous solubility of the drug. Specific excipient method focuses on selecting specific excipients such as water dissoluble Ca Salt, specific dis-integrant combinations for preparing fast dissolving tablets. Earlier reports and references favours its path that solvent deposited systems of water insoluble drug using inert excipients increases the solubility and make a way for faster dissolution[4,5,6,7,8.]

Addition of dis-integrants in faster release formulations leads to quick disintegration and hence improves dissolution. Microcrystalline cellulose, substituted hydroxy propyl cellulose,

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cross linked PVP though water insoluble, absorbs water and swells due its capillary action, and considered as effective dis-integrant.

Advantages of Faster Release Formulation[9]:

1. Ease of Administration
2. Improved patient compliance
3. Requires no/less water intake
4. Quick disintegration and dissolution of dosage form
5. Overcomes unacceptable taste of drugs
6. Allows high drug loading
7. Pleasant mouth feel

The objective of present study was to investigate the possibility of improving the solubility and dissolution rate of nimesulide by formulating solvent deposition systems of Nimesulide using inert excipients like starch, Aerosil, Microcrystalline cellulose in the ratio (1:2) and (1:4). Drug content was estimated spectrophotometrically at 394nm by extracting the drug with Methanol. In addition, the invivo anti inflammatory activity were also investigated and reported.

Experimental work done:

Materials:

Nimesulide (gift sample from) Dr. Reddy's Lab

Starch	:	Chemspure Madras.
Aerosil	:	Scientific Supplies Trichy
Microcrystalline Cellulose	:	Scientific Supplies Trichy
Potassium Di Hydrogen Phosphate	:	Fischerinorganics & Aromatics
Sodium Hydroxide	:	Fischerinorganics & Aromatics
Acetone	:	Fischer Inorganics & Aromatics

Methods:

Formulation of SD systems of Nimesulide

Nimesulide faster release formulation includes STARCH, Aerosil, Microcrystalline Cellulose in the ratio 1:2 and 1:4 solvent deposited system are prepared by dissolving Nimesulide in acetone to produce a clear solution. The excipient was dispersed in the solution by stirring. The solvent was then removed by evaporation at 40ºC with continuous stirring. The

resultant mass is then dried, pulverized and passed through Mesh no: 100 formulations were stored in air tight containers until evaluation.

Estimation of Drug Content:

The Drug content of solvent deposited systems of Nimesulide was estimated as per the method described by Chowdary and Ramesh[10]. Accurately weighted quantity of 100mg of SD formulation of Nimesulide were extracted with methanol in 100ml standard Flask. The extracted formulation was diluted suitably with Methanol and the drug content was estimated by using shimadzu UV 150-02 spectrometer at 394nm and the results are tabulated in Table I.

In Vitro Dissolution Studies:

The dissolution rate of Nimesulide in pure form and in solvent deposited formulations was studied in 900ml of phosphate buffer pH 7.4 as dissolution medium using Rotary – Basket USP XXI. Dissolution apparatus at 37±0.5ºC, a speed of 100rpm were used in each test. Formulations were placed in Rotary basket and it is allowed to rotate at constant speed. Samples are withdrawn at different time intervals. Filtered, diluted suitably and analyzed for the drug content spectrometrically at 394nm, and the results are tabulated in Table II.

***In Vivo* Studies:**

Anti-inflammatory activity of SD formulations of Nimesulide by carragenan induced pawoedema method was carried out in albino rats as per the method described by klinteret.al[11,12] Albino rats of either sex weighting about 150-200gms were divided into eight groups of four animals each. 0.1ml of carragenan in 1% w/v in normal saline was injected into sub plantar region of hind paw of the group of animals. Animals received normal saline was treated as control. Normal saline and all other drug formulations were administered orally in the dose of 6gm/150gm of body weight of albino rats, one hour before carragenan treatment the volume of hind paw oedema was measured by plethysmometer at the interval of 1,2,3 hours Mean increases in paw volume, percentage inhibition of inflammation swelling was calculated. The percentage inhibition of paw volume produced by the different formulation of Nimesulide was compared with that of control. Percentage inhibition was calculated by using the formula:

Percentage inhibition: $100\ (1\text{-}V_t / V_o)$

V_t is the paw volume of control (saline treated animals) and the results are presented in Table III.

Stability studies of SD formulations:

SD formulations of Nimesulide were taken and started in separate containers, and it is exposed to different temperature for 30 days. Samples were withdrawn at different time intervals extracted with methanol and analyzed spectrophotometrically at 394nm for drug content and the results are tabulated in Table 1V

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Interaction studies:

The interaction between the drug and added excipients (Starch, Avicel, MCC) in SD formulation of Nimesulide was analyzed by IR studies. Reports of IR Reveals that there is no interaction by drug and excipients.

Results and Discussions:

Faster release formulation of Nimesulide by solvent deposited technique was formulated by using starch, microcrystalline cellulose in the drug carrier ratio1:2, 1:4. The drug content estimation confirms the increased aqueous solubility of the drug and the proportionality changes of solubility with drug carrier ratio, and improvement in vitro dissolution confirms the increased aqueous solubility and the results are tabulated in Table 1 to 4. Release rate of drug from the carrier exhibits following order.

Microcrystalline cellulose > Aerosil > Starch

IR spectral studies confirm that there is no interaction between drug and excipients. In vivo anti-inflammatory studies may by carrageenan induced paw volume confirms anti-inflammatory activity of solvent deposited faster Release formulation of Nimesulide (Table 5).

Aqueous solubility studies of nimesulide in different concentration of carrier's solubility of nimesulide in distilled water is 2.59 µg/ml

Sr. No.	Name of the carrier	Solubility of Nimesulide in different concentration of carriers					
		0.2%	0.4%	0.6%	0.9%	1.2%	1.5%
1	Starch	10.70	13.35	16.25	19.63	24.80	29.17
2	Aerosil	12.29	16.82	20.73	23.41	27.56	32.92
3	M.C.C.	17.68	20.14	22.45	25.12	30.79	35.38

Table 1: Standard curve

Sr. No.	Concentration µg/ml (x)	Absorbance (y)
1	5	0.218
2	10	0.439
3	15	0.654
4	20	0.877
5	25	1.090

Table 2: Dissolution data for pure nimesulide in buffer pH 7.4

Sr. No.	Time in Minutes	Concentration µg/ml	% of drug Release
1	15	15.25	13.72
2	30	25.20	22.61
3	60	34.33	30.90
4	90	40.50	36.36
5	120	52.16	47.04
6	180	80.50	72.45

Table 3: Dissolution data for solvent deposited systems in Nimesulide in buffer pH 7.4

Sr. No.	Drug + Carrier Ratio	Percentage of Drug Release					
		15 min	30 min	60 min	90 min	120 min	180 min
1	Drug + Star 1: 2	19.31	25.83	2.72	47.36	58.47	81.15
2	Drug + Starch 1:4	22.52	28.60	33.05	50.24	62.30	85.98
3	Drug + Aerosil 1:2	25.29	32.57	39.65	53.56	65.33	88.57
4	Drug + Aerosil 1:4	29.81	36.13	42.40	27.20	69.90	91.86
5	Drug + MCC 1.2	32.12	38.51	46.23	63.82	73.03	95.15
6	Drug + mcc 1:4	35.60	42.36	57.83	64.07	77.52	97.38

Table 4: Estimation of Drug content in the formulation 1:2 (33.5 gms : 66.5 gms) Ratio

Dissolution data for pure Nimesulide in Buffer pH 7.4

Sr. No.	Drug / Excipient	Amount of Nimesulide in grams		% of Nimesulide	
		1:2	1:4	1:2	1:4
1	Drug + Starch	29.96	18.28	89.48	92.83
2	Drug + Aerosil	31.41	17.90	94.75	93.12
3	Drug+ MCC	32.11	19.18	96.50	88.30

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Table 5: *in vivo* studies

Sr. No.	Name of the expicient + Drug	Mean paw volume (Time in Minutes)				% inhibition (Time in Minutes)			
		30	60	120	180	30	60	120	180
1	Control	0.89±0.13	0.92±0.10	0.95±0.14	0.99±0.09	-	-	-	-
2	Starch + Drug (1:2)	0.70±0.09	0.68±0.14	0.64±0.12	0.59±0.14	21.05	25.57	32.36	40.25
3	Aerosil + Drug (1:2)	0.66±0.09	0.63±0.10	0.60±0.13	0.54±0.009	25.74	30.95	36.82	44.32
4	MCC + Drug (1:2)	0.63±0.12	0.61±0.14	0.52±0.11	0.50±0.13	29.13	33.16	43.40	49.04
5	Starch + Drug (1:4)	0.60±0.10	0.58±0.12	0.50±0.09	0.46±0.11	30.25	36.20	47.13	53.15
6	Aerosil + Drug (1:4)	0.56±0.13	0.55±0.09	0.48±0.10	0.42±0.12	36.43	40.00	49.06	56.95
7	MCC + Drug (1:4)	0.52±0.11	0.50±0.13	0.44±0.12	0.38±0.10	40.96	45.17	53.49	61.35

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PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITY OF PETROLEUM ETHER, METHANOL AND ACETONE EXTRACTS OF *CATHARANTHUS ROSEUS* LEAVES AND FLOWERS

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Abstract:

Catharanthus roseus is commonly known by its different regional names in India as , Ainskati, Billaganneru, Nayantara, Nityakalyani, Periwinkle, Rattanjot, Sada bahar, Sadaphul, Ushamanjairi etc. This is one of the medicinal plants which belong to the magnoliopsida class and apocynaceae family (Dog-bane family). This ornamental is cultivated in many public garden places as well as in botanical gardens of schools, colleges, universities and national research centers worldwide. This evergreen plant has been described as erect, bushy, ever blooming and self-pollinating herb. It's stem and leaves contain white latex. Alkaloids have been abundantly found in *Catharanthus roseus* and hence it has got immense medicinal importance. Traditionally, leaves of this plant had been used as crude medicine for the treatment of cancer, diabetes and many ailments. Nowadays, it has been looked as a very important source of new lead compounds for drug discovery and development. In the present investigation, we have performed phytochemical screening and evaluated antibacterial activity of selected organic solvent extracts of *Catharanthus roseus* leaves and flowers. Antibacterial activity was recorded against selected clinical pathogens viz. *Escherichia coli*, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis.* The bioactive compounds present in *Catharanthus roseus* may have the tremendous applications in the field of medical biotechnology and chemoinformatics. Conventional use and scientific observations of plants for medicinal purposes provide the basis for such type of investigation.

Keywords: *Catharanthus roseus*, phytochemicals, qualitative analysis, antibacterial activity

Introduction:

Most of the herbal plants are used as therapeutic tools for the treatment and prevention of many diseases. Medicinal plants contain bioactive compounds which can exhibit physiological effects due to presence of phytochemicals include alkaloids, flavonoids, carbohydrates, terpenoids, steroids, tannins (Chinnavenkataraman and Srinivasan, 2012). Chemotherapeutic potential of this plant has been well described by Cragg and Newman (2005) and MacLaughlin *et al.* (2006). Leaves of *C. roseus* are used extensively in folk medicine for decreasing sugar level of blood hence showed significant anti-hyperglycemic effect; moreover oil extracted from these leaves have shown antibacterial activity (Aziz *et al.,* 2014). *Catharanthus roseus* widely grows in the Indian subcontinent. This ornamental plant belongs to apocynaceae family in which plants generally grow up to 75 cm. The stem produces milky latex. In average, 2.5 to 9 cm long and 1.5 to 4 cm wide leaves are present in pairs on stem of this plant. A variety in flower colour has been observed as white petals with a yellow or crimson spot and lavender-pink petals with a crimson spot at the center. In general, *Catharanthus roseus* has two common flower colors as pink (Rosea) and white (Alba).

Traditionally, leaves of *Catharanthus roseus* were used to cure various diseases. Ross (2003) has reported use of this plant traditionally for medication purpose with variety of techniques and combinations in many countries, for example, Australia, Brazil, China, Cook Islands, Dominica, England, Europe, France, French Guiana, India, Jamaica, Kenya, Mexico, Mozambique, North Vietnam, Pakistan, Peru, Philippines, South Africa, South Vietnam, Taiwan, Thailand, Venda, and West Indies. Ross (2003) has listed more than 200 chemical constituents from *Catharanthus roseus.* Different extracts and formulations based on this plant have shown many pharmacological activities and effects such as abortifacient effect, acid phosphatase inhibition, acid phosphatase stimulation, alkaline phosphatase stimulation, alkylating activity reduction, animal repellent activity, antiascariasis activity, antibacterial activity, antidiuretic activity, antifertility effect, antifungal activity, antihypercholesterolemic activity, antihyperglycemic activity, antihypertensive activity, anti-inflammatory activity, antimalarial activity, antimitotic activity, antimutagenic activity, antineoplastic activity, antispasmodic activity, antispermatogenic effect, antitumor activity, antiviral activity against plant pathogens, cardiotonic activity, CNS depressant activity, cytotoxic activity, glutamate pyruvate transaminase inhibition effect, hyperglycemic activity, hypoglycemic activity, hypotensive activity, inotropic effect (negative), insect feeding deterrent, insect sterility induction, insecticidal activity, insulin activity, larvicidal activity, leukopenic activity, peroxidase activity, phospholipase c activity, smooth muscle relaxant activity, spasmogenic activity, toxic effect

(general), uterine relaxation effect, uterine stimulant effect and weight loss effect (Ross, 2003). Hence, *Catharanthus roseus* have been looked as a very important source of new lead compounds for drug development and discovery (Gajalakshmi *et al.*, 2013).

In present investigation, we have performed a comparative study to determine the effect of selected solvents viz. petroleum ether, methanol and acetone on the extraction yield. Furthermore, growth inhibitory activity of the obtained extracts was evaluated against selected opportunistic pathogens.

Materials and methods:

Collection of the plant material and preparation of powdered samples

Healthy and fresh plants of *Catharanthus roseus* were collected from local nursery and brought to the laboratory (Figure 1). All leaves and flowers were excised by using a scalpel. The excised materials were washed with distilled water and kept for drying under shaded place. The dried leaves and flowers were pulverized by using a mechanical blender and sieved individually to form the fine powder which was then stored separately in air tight bottles for further use.

Figure 1: The medicinal and ornamental *Catharanthus roseus* plant selected for the phytochemical analysis

Preparation of extracts

Powdered form of leaves and flowers of *C. roseus* sample (1 g) were immersed individually in different solvents (petroleum ether, methanol, and acetone) at a sample: solvent ratio of 1: 20 (w/v) for 24 h at 60°C. The mixtures were then homogenized at 60°C for 4 h using a homogenizer and then filtered. The filtrates were concentrated at 60°C using a rotary evaporator, freeze-dried for 24 h and stored at 4°C prior to perform further experiments (Truong *et al.*, 2019).

Calculation of extraction yield

Following formula was used to calculate extraction yield (Truong et al., 2019).

Extraction yield (%) = [(Weight of the extract after evaporating solvent and freeze drying) / (Dry weight of the sample)] X 100

Qualitative screening for the selected phytochemicals

Freeze-dried extracts of leaves and flowers of *C. roseus* were used for the qualitative screening of selected phytochemicals (Aziz *et al.*, 2014).

(i) Test for alkaloid

Selected extracts were warmed separately with 2% H_2SO_4 for two min. It was filtered and few drops of Dragendroff's reagent were added, for the detection of alkaloids. The contents were observed for the presence or absence of red precipitation.

(ii) Test for flavonoid

Extracted samples were heated with 10 mL of ethyl acetate in boiling water bath for 3 min and then filtered. 1 mL of dilute ammonia solution (1%) was added in each filtrate and then shaken well till the formation of layers. Ammonia layer was observed for the presence or absence of yellow coloration.

(iii) Test for carbohydrate

The extracts were shaken well with water and then filtered. Then 6 drops of Molisch's reagent were added to the aqueous filtrates and again shaken well. Then 1 mL of concentrated H_2SO_4 (1 mL) was carefully added in each test tube and the observations were recorded.

(v) Test for terpenoids (Salkowski test)

2 mL of chloroform was added in extract-samples and the test tubes were shaken. Later on, 3 mL of concentrated sulphuric acid was added carefully and mixed well by mild shaking. Then, observations were recorded.

(vi) Test for protein and amino acid

Extract-samples were added in 10 mL of distilled water, dissolved well and filtered. The filtrates were subjected for biuret and ninhydrin tests.

(a) Protein detection by Biuret test: One drop of 2% copper sulphate solution was added in each 2 mL of filtrate. Then 1 mL of ethanol (95%) was added to it followed by excess potassium hydroxide pellets. The observation in ethanolic layer was recorded.

(b) Detection of amino acid by Ninhydrin test: Two drops of ninhydrin solution were added to 2 mL of aqueous filtrate. Then the observations were recorded on the basis of either presence or absence of a characteristic purple color.

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(vii) Analytical test for reducing sugar

1 ml of selected plant extract was added in 1ml of Fehling's solution A and B, and then boiled for few minutes. The content was observed for the presence or absence of red color at the bottom.

Screening for antibacterial activity of selected plant extracts

The agar well diffusion method was used to examine antibacterial activity of selected plant extracts. 20 µL of selected pure bacterial culture of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* was added in each nutrient agar plate individually and spread uniformly by using a glass spreader. Agar well of 5 mm in diameter was prepared in each plate with the help of a sterilized stainless cork borer. The plates were labeled appropriately. In each well 10 µL of selected plant extract was added using a micro-pipette. Antibiotic neomycin (25 µg/disc) was used as control against selected bacteria. All the plates were incubated at 37°C for 24 hours. Each plate was observed for the presence or absence of growth inhibition zone (Anosike *et al.*, 2012; Rathod *et al.*, 2019 [a,b,c]).

Results and Discussion:

Analysis of extraction yield

Results have shown a difference in the extraction yield as different solvents used. Among the different solvents tested, methanol has resulted in the greatest extraction yield (35.12%), followed by acetone (29 %) and petroleum ether (12 %) (Figure 2). This has indicated that, the extraction efficiency favored the highly polar solvents.

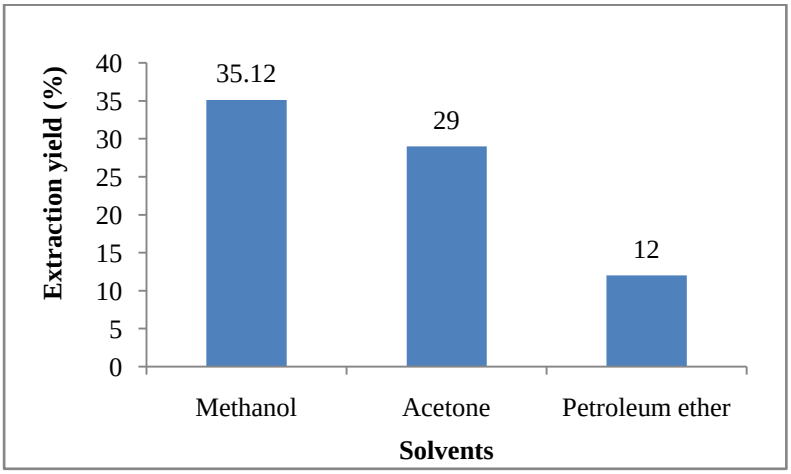

Figure 2: Effect of selected organic solvents on extraction yield

Qualitative screening for selected phytochemicals

(i) Test for alkaloid

Red precipitation was observed in the test tube, hence presence of alkaloid was confirmed in leaves and flower extracts of *C. roseus*. Alkaloids are a group of nitrogen containing natural compound. Many alkaloids have been used from a long time in medication (Roy, 2017).

(ii) Test for flavonoid

Yellow coloration was observed in the ammonia layer hence presence of flavonoid was confirmed in leaves and flower extracts of *C. roseus*. Flavonoids are medicinally important since they exhibit antioxidant property and produce many other beneficial effects which make them suitable candidates in therapeutics.

(iii) Test for carbohydrate

A brown ring was observed at the interface hence presence of carbohydrate was confirmed in leaves and flower extracts of *C. roseus*. Carbohydrates are produced by photosynthesis and abundantly found in nature. They have the unique role in plant metabolism (Asif *et al.,* 2011).

(v) Test for terpenoids (Salkowski test)

Reddish brown colour was developed at the interface in the raction mixture of flower extract but not developed in leaves extract; hence presence of terpenoid was confirmed only in flower extract of *C. roseus*. Terpenes have many applications in food and pharmaceutical industries. Plant oils, which contain terpenes, have shown inhibiting multiple species of bacteria e.g., cinnamon oil has shown broad-spectrum activity against *Pseudomonas aeruginosa* as reported by Zwenger and Basu (2008).

(vi) Test for protein and amino acid.

(a) Biuret test for protein detection:

Purple colour was observed in the tube hence presence of protein was confirmed in leaves and flower extracts of *C. roseus*.

(b) Ninhydrin test for amino acid detection:

A characteristic purple colour was observed in the reaction mixture hence presence of protein was confirmed in leaves and flower extracts of *C. roseus*.

(vii) Test for reducing sugar

Red color was observed at the bottom of reaction mixture hence presence of reducing sugar was confirmed in leaves and flower extracts of *C. roseus* (Table 1).

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Table 1: Phytochemical analysis of *Cathranthus roseus* leaves and flower

Name of the photochemical	Petroleum ether, methanol, and acetone extract	
	Leaves	**Flower**
Alkaloids	Present	Present
Flavonoids	Present	Present
Carbohydrates	Present	Present
Terpenoids	Absent	Present
Protein	Present	Present
Amino acid	Present	Present
Reducing sugar	Present	Present

Screening for antibacterial activity of selected plant extracts

Remarkable antibacterial activity was shown by all prepared extracts of *C. roseus* (Table 2). The bioactive compounds in the organic solvent extracts of leaves and flowers of *C. roseus* have antimicrobial potency and perhaps may have contributed to its antimicrobial activity. The methanolic extracts of leaves and flowers of *C. roseus* have shown the highest antibacterial activity than acetone and petroleum ether extracts but the activity of these extratcts cannot be overlooked as they do exhibit some antibacterial activity.

Table 2: Screening for antibacterial activity of organic solvent extracts of leaves and flowers of *C. roseus*

Test culture	Petroleum ether extract		Acetone extract		Methanol extract	
	Leaves	**Flowers**	**Leaves**	**Flowers**	**Leaves**	**Flowers**
P. aeruginosa	07	06	09	08	10	09
B. subtiIis	09	08	08	07	11	10
S. aureus	09	08	06	06	10	09
E. coli	08	08	08	07	11	09

(Diameter of growth inhibition zone has recorded in mm and size of cork borer was 5 mm in diameter)

Conclusion:

Secondary metabolites viz. alkaloid and flavonoid were found in selected organic solvent extracts of leaves and flowers of *C. roseus.* These extracts have shown remarkable antibacterial

activity. The plant studied here can be used as a potential source of useful bioactive compounds which may have medicinal uses and the therapeutic values. We therefore, suggest further characterization of these bioactive compounds to obtain useful chemotherapeutic agents. Purified phytochemicals from *C. roseus* can be used in pharmaceutical approaches for better medication.

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