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ANTI-INFLAMMATORY AND ANTIBACTERIAL POTENTIAL OF *CARISSA CARANDAS* AND *CARISSA SPINARUM* NANOEMULSIONS

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Keywords:

Carissa carandas, *Carissa spinarum*, Nanoemulsion, Anti-inflammatory activity, Well diffusion method, Proteinase activity

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ABSTRACT: The studies in the present article aims at developing an optimal oral polyherbal nanoemulsions from *Carissa carandas* (CC) and *Carissa spinarum* (CS) which are noticed in ethnobotanical herbarium for their pharmacological relevance. Nanoemulsions were developed in different proportions of oil and surfactant systems in order to enhance its oral bioavailability. Solubility studies were carried out for the preparation of the formulation. Pseudoternary phase diagrams were constructed by aqueous titration technique and different polyherbal nanoemulsions were prepared. Formulations selected from o/w nanoemulsion region were subjected to various thermodynamic stability tests. Optimized formulations were evaluated for physicochemical tests. *In vitro* anti-inflammatory activity was carried out by inhibiting heat induced albumin denaturation, proteinase activity, red blood cells membrane stabilization and broth dilution assay. Phase diagram with 1:2 S/Smixhas shown largest area of nanoemulsion. *In vitro* dissolution studies revealed that release of drug from nanoemulsion 2 (96.88±1.29%) was faster than the nanoemulsion1 (85.36±2.12%) in 120 min. The formulation showed satisfactory results for *in vitro* anti-inflammatory activity by inhibiting the heat induced albumin denaturation and red blood cells membrane stabilization as well as significant inhibition for proteinase activity at 600µg/ml. The NE2 showed excellent antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Salmonella typhi* whereas NE1 against *Escherichia coli.* These studies indicate that the nanoemulsion may be used as a promising drug delivery vehicle for bioavailability enhancement of hydrophobic drugs.

INTRODUCTION: Medicinal herbs have immense potential as therapeutic aid. They have played a vital role in combating not only multiple diseased conditions but also served as potential material for maintaining proper health condition. Undoubtedly, molecules derived from natural sources, including plants, marine organisms and microorganisms, have played, a superior role in the discovery of leads for the development of conventional drugs for various diseases.

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The active constituents of herbal drugs shows synergistic action and enhance therapeutic value which are having complex structure. These constituents include tannins, flavonoids and terpenoids when incorporated into formulation showed increased bioavailability at low therapeutic dose [1]. The plants selected for designing of the formulation belongs to the family of Apocynaceae, a genus of 32 species out of which 8 species are enlisted in Indian Literature.

Carissa carandas and *Carissa spinarum* are of economic importance. The fruits of these plants are edible and widely known in tribal communities in India. These plants have been proven for anticonvulsant, cardiotonic, hepatoprotective, antimicrobial, antiviral, antidiabetic, cancer and immunomodulatory utilities [2 - 5].

We have attempted to formulate plant extracts in the form of nanoemulsion that are similar to conventional pharmaceutical dosage and are thought to be transparent or translucent systems containing droplets with a mean diameter of 100-500 nm [6]. Nanoemulsions are good candidates for oral delivery of poor water insoluble drugs because of the surfactant induced permeability. Nanoemulsions incorporate the hydrophobic drug into the oil phase thereby increasing its solubility and enhance gastrointestinal tract absorption [7-9].

In this context, the present study aimed at developing and evaluating optimal nanoemulsions from plant extracts using minimum surfactant concentration, so that nanosized droplets could be maintained on dilution by the GI fluids with an aim to increase its bioavailability. The dose of herbal extract varied between 250 mg to 500 mg. Nanoemulsions containing oil, surfactant and cosurfactant were developed with help of phase titration method and subjected to *in vitro* anti-inflammatory and antibacterial screening.

MATERIALS AND METHODS:

Collection, authentication and extraction: The fruits of *Carissa carandas* (CC) and *Carissa spinarum* (CS) fruits were collected in month of June - July from the local market, authenticated at Agharkar Research Institute (No.3/187/2013/Adm. 1692) and air-dried at room-temperature between 28-30 °C. The dried fruits were crushed in mortar and pestle to get coarse powder and treated with petroleum ether extraction. Soxhlet extraction was carried out at 40-60 °C till the thimble became colorless separately for each plant species. The round bottom flask contents were mixed of both the extracts and dried in vacuum. The powder obtained was subjected to further studies.

Materials: Oleic acid, almond oil, sesame oil and olive oil were purchased from Kamani Oil Industries Ltd. (Mumbai, India). Castor oil, isopropyl myristate, arachis oil, tween 20, tween 80, brij 35 and poloxamer 407 were purchased from Loba Chemie Pvt. Ltd. Cremophore RH 40, Capryol 90 and Transcutol P were purchased from Gattefosse India Pvt. Ltd. Isopropyl alcohol, propylene glycol and ethanol were purchased from S. D. Fine Chemicals (Mumbai, India). Dialysis bags (Molecular weight cut - off (MWCO), 12000g/mol) were procured from Hi Media, India. All other chemicals were of analytical grade used in the studies.

The microorganisms used for these studies include *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi*, *Escherichia coli* were obtained from Department of Microbiology, Vivekanand Education Society's college of Arts, Science and Commerce, Mumbai, India.

Preparation of the nanoemulsion: [10]

Solubility Studies: For the preparation of nanoemulsion, various oils were evaluated for the solubility of the drug in the oils. An excess amount of drug was added in 2 ml of each oil separately in 5 ml capacity stoppered vials, and mixed using a vortex mixer. These vials were then kept at 25±1.0 ºC in an orbital shaker for 72 hours to reach equilibrium. The equilibrated vials were removed from shaker and centrifuged at 15000 rpm for 15 min using centrifuge. The supernatant was taken and filtered through a 0.45 µm membrane filter. The concentration of drug was determined in different oils by using UV-VIS Spectrophotometer at detection wavelength of 275.5 nm.

Pseudoternary phase diagram study: The pseudoternary phase diagrams consisting of oil, S/Smix (surfactant-co-surfactant mixture) and double distilled water were developed using the aqueous titration method. Surfactant and cosurfactant were mixed in different volume ratios (1:1, 1:2, 1:3, 2:1 and 3:1) selected on the basis of increasing concentration of cosurfactant with respect to surfactant and vice versa. For each phase diagram, oil and a specific S/Smix ratio was mixed properly in different volume ratios. Ten different combinations of oil and S/Smix, (1:1, 1:2, 1:3,1:4, 1:5, 1:6, 1:7, 2:1, 2:2, 3:1) were slowly titrated with aqueous phase and visually inspected for transparency and flowability.

Pseudoternary phase diagrams were developed using aqueous titration method by adding drop by drop external aqueous phase under gentle vortexing to each oil and S/Smix ratio (spontaneous emulsification technique). Visual observation was carried out for transparency and easily flowability of o/w nanoemulsions. The physical state of the nanoemulsion was marked on the phase diagrams

with three axis representing an aqueous phase, oil and S/Smix. For each phase diagram, nanoemulsion area was plotted and the wider region indicated the better self-nanoemulsifying efficiency.

Characterization of prepared Nanoemulsion: [11]

Thermodynamic stability: In order to find out the stable nanoemulsion, the nanoemulsions were subjected to following thermodynamic stability studies.

i) **Centrifugation:** The formulations were centrifuged at 3000 rpm for 30min. Those formulations that did not show phase separation were subjected to freeze thaw studies.
ii) **Freeze thaw cycle:** Nanoemulsions were kept in deep freezer (at -20 ºC) for 24 h and then at room temperature. The thermodynamically stable nanoemulsions returned to their original form within 2-3 min. 2-3 such cycles were repeated.
iii) **Heating cooling cycle:** Six cycles between refrigerator temperature (4 ºC) and 40 ºC with storage of 48 h were performed. Those formulations which were stable at these temperatures will be subjected to further studies.

Particle size and Zeta potential analysis: The average droplet size and polydispersity index (PDI) of nanoemulsions were determined by photon correlation spectroscopy using Malvern Zetasizer (Nano ZS-90, UK).

Viscosity determination: The rheological property of the formulations was determined as such without dilution using Brookfield DV-II ultra^{+} viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) using spindle # CPE 40.

Refractive index: The refractive index of the system was measured by an Abbe refractometer by placing a one drop of nanoemulsion on the slide in triplicate.

Drug content: The drug content was calculated by UV visible spectrophotometer. The formulation was diluted to required concentration using methanol as solvent and the absorbance was measured at 275.5nm against a solvent blank.

***In vitro* drug release:** The quantitative *in vitro* release test was performed in 500ml of Phosphate buffer pH 6.8 using USP Dissolution apparatus Type II at 75 rpm and 37±0.5 °C using dialysis bag technique. Sampling was done at different time intervals and analyzed by UV-visible spectroscopy at 275.5 nm.

Screening of nanoemulsion for *in vitro* anti-inflammatory activity:

Inhibition of albumin denaturation: The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of the nanoemulsions so that final concentrations become 100, 200, 300, 400, 500, 600 and 1000 µg/ml. Similar volume of double-distilled water was used as control. Then the mixtures were incubated at 37±2 °C in a BOD incubator for 15min and then heated at 70 °C for 5 min. After cooling, their absorbance was measured at 660 nm (SHIMADZU, UV 1800) by using vehicle as blank. Diclofenac sodium at the final concentration of (100, 200, 300, 400, 500, 600, 1000 µg/ml) was used as reference drug and treated similarly for determination of absorbance. The percentage inhibition of protein denaturation was calculated [12].

Percentage inhibition of protein denaturation =

$$\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} X\ 100$$

Proteinase inhibitory action: The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mMTris HCl buffer (pH 7.4) and 1 ml of different concentrations of nanoemulsions. The mixture was incubated at 37 °C for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated[12].

Percentage inhibition of proteinase inhibitory activity =

$$\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} X\ 100$$

Membrane stabilization test: Fresh whole human blood (10ml) was collected from local blood bank, Mumbai and transferred to heparinized centrifuged

tubes. The tubes were centrifuged at 3000 rpm for 10 min were washed 3 times with equal volume of normal saline and reconstituted as 10% v/v suspension with normal saline.

The reaction mixture (2 ml) consisted of 1 ml of test sample and 1 ml of 10% RBCs suspension, instead of drug only saline was added to the control test tube. Aspirin was taken as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in a water bath at 56 °C for 30 min. At the end of the incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates. Percentage inhibition of membrane stabilization activity was calculated [12].

Percentage inhibition of membrane stabilization activity =

$$\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} X\ 100$$

Evaluation of nanoemulsion for anti-microbial activity: Antibacterial properties of developed nanoemulsions (NE 1 and NE 2) and standard drug (Tetracycline) were studied by well diffusion method. Overnight grown culture of all strains were inoculated in MRS broth and incubated for 18-20 h at 37 °C. The grown cultures were harvested by centrifugation at 10000 g for 10 min at 4 °C and 200 µl cell-free supernatants were used for the assay. Bacterial isolates were studied for minimum inhibitory concentration activity against standard pathogens (*E. coli* MTCC1687, *Bacillus subtilis* ATCC9372, *S. aureus* ATCC 25923 and *S. typhi* MTCC531). Indicator strains of 300 µl were mixed properly in 10 ml of Luria Broth (LB) soft agar and overlaid on LB-base plate and left for solidification. After solidification, the holes were bored on a plate with the help of pipette tips (200µl, diameter 0.7 cm).

Then the holes were sealed by adding 1-2 drops of soft agar in order to avoid leakage of the supernatant. 100 µl of prepared supernatant was added into each well and kept in the fridge for 1-2 h for diffusion of the supernatant. The assay plates were incubated for 6-7 h at 37 °C. The zone of inhibition was measured with the help of zone measuring scale [13].

Statistical analysis: The data collected during the course of the experimentation was subjected to significance testing using mean ± standard deviation (SD) analysis. Statistical significance was set at $P<0.05$. Results were denoted as mean± standard deviation (SD) of triplicate experiments.

RESULTS AND DISCUSSION:

Extraction Yields: *Carissa carandas* and *Carissa spinarum* fruits were having yield of 13.12 and 14.44 % respectively.

Preparation of the nanoemulsion:

Solubility studies: The solubility of drug was found to be 89.35mg/ml in oleic acid, which was highest among the tested oils. Tween 80 and ethanol showed highest solubility of drug *i.e.* 64.36 mg/ml and 92.52 mg/ml, respectively, among the surfactants and cosurfactants studied (**Table 1**).

TABLE 1: SOLUBILITY PROFILE OF DRUG IN OILS, SURFACTANTS, AND COSURFACTANTS (n=3)

Sr.no.	Oil, Surfactant, Cosurfactant	Solubility (mg/ml)
1.	Oleic acid	89.35 ± 1.63
2.	Almond oil	32.02 ± 2.02
3.	Castor oil	65.69 ± 1.33
4.	IPM	52.78 ± 1.08
5.	Olive oil	23.65 ± 2.14
6.	Arachis oil	10.33 ± 0.59
7.	Seasame oil	8.23 ± 0.98
8.	Tween 80	58.32 ± 1.74
9.	Tween 20	64.36 ± 1.69
10.	Brij 35	52.39 ± 1.33
11.	Cremophore RH 40	7.89 ± 2.65
12.	Span 20	14.2 ± 1.3
13.	Poloxamer 407	12.37 ± 0.25
14.	Capryol 90	8.36 ± 1.98
15.	Ethanol	92.52 ± 1.23
16.	Isopropyl Alcohol	87.35 ± 1.36
17.	Propylene glycol	42.2 ± 1.87
18.	Transcutol P	10.36 ±2.54

Construction of pseudoternary phase diagram: Phase diagrams were constructed in the absence of extracts to identify nanoemulsifying regions which helped to optimize the concentration of oil and surfactants in nanoemulsion. Phase diagram with 1:2 S/Smix had largest area of nanoemulsion formation (Figure 1). Various blends were selected from pseudoternary phase diagram, the formulation in which the amount of oil phase was completely solubilized the drug and could accommodate the optimum quantity of S/Smix and distilled water were selected.

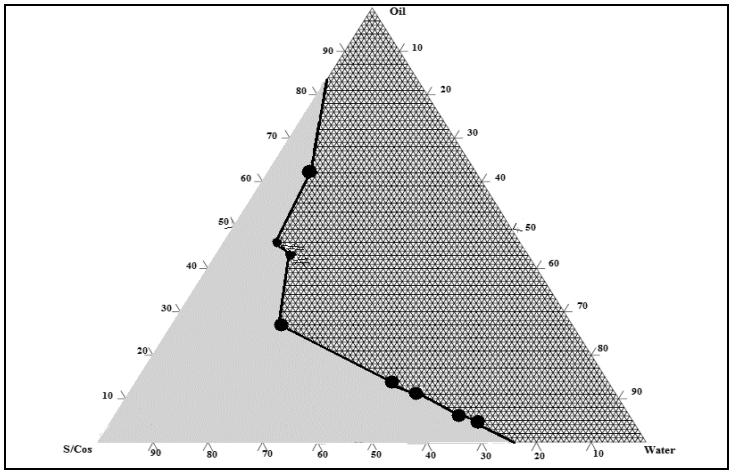

FIG. 1: PSEUDOTERNARY PHASE DIAGRAM WITH S/Smix 1:2

Characterization of prepared Nanoemulsion:

Thermodynamic Stability: Nanoemulsions can be differentiated from ordinary emulsions due to their thermodynamically stability[14 - 15]. In order to avoid phase separation, creaming or cracking, stability tests like centrifugation and freeze thaw cycle were performed. The results of Centrifugation, Freeze Thawing cycle and heating cooling cycle are observed in **Table 2**.

TABLE 2: THERMODYNAMIC STABILITY SCREENING OF DIFFERENT FORMULATIONS SELECTED FROM PHASE DIAGRAMS

S/Smix ratio	Oil (%v/v)	S/Smix (%v/v)	Aqueous (%v/v)	Centrifugation	Freez	H/C	Inference
1:1	10	30	60	√	√	√	Passed
	10	35	55	√	√	√	Passed
	10	45	45	√	√	√	Passed
	12	35	53	√	√	X	Failed
	15	12	73	X	√	X	Failed
1:2	10	16	74	√	√	X	Failed
	10	40	50	√	√	√	Passed
	15	40	45	X	√	√	Failed
	20	27	53	√	√	X	Failed
1:3	10	23	67	√	√	√	Passed
	10	40	50	√	√	√	Passed
	15	44	41	√	√	X	Failed
	20	48	32	√	√	X	Failed
2:1	10	35	55	√	√	√	Passed
	10	40	50	√	√	√	Failed
	15	42	43	X	√	X	Failed
	10	45	45	√	√	√	Passed
	20	30	50	√	X	√	Failed
3:1	10	35	55	√	√	√	Passed
	10	45	45	√	X	X	Failed

Freez= Freez-thaw cycle, H/C= Heating cooling cycle

Particle size and Zeta potential analysis:

Droplet size has been found to affect the degree of drug absorption, the smaller the droplet size, the larger the interfacial area for drug absorption. The minimum droplet size was observed in NE2 may be attributed to the composition of S/Smix. In case of NE2, the % of surfactant in S/Smix was higher as compared to NE1 formulations. The higher droplet size was observed in case of NE1 compared to NE2 which may be due to the expansion of the interfacial film by the cosurfactant leading to increased droplet size. The PDI of all the formulations was less than one, indicating narrow size distribution. NE2 formulation has smallest particle size (177.2nm) (**Fig. 2**) which was significant in comparison to NE 1. The surface charge also plays an important role in the stability of the nanoemulsion and the magnitude of zeta

potential is an indicative of the colloidal stability of the system. The highly negative zeta potential indicates that the particles are electrically stabilized to resist aggregation. The highly negative zeta potential indicates that the formulation has particles which are good and resist to the process of aggregation (**Fig. 3**).

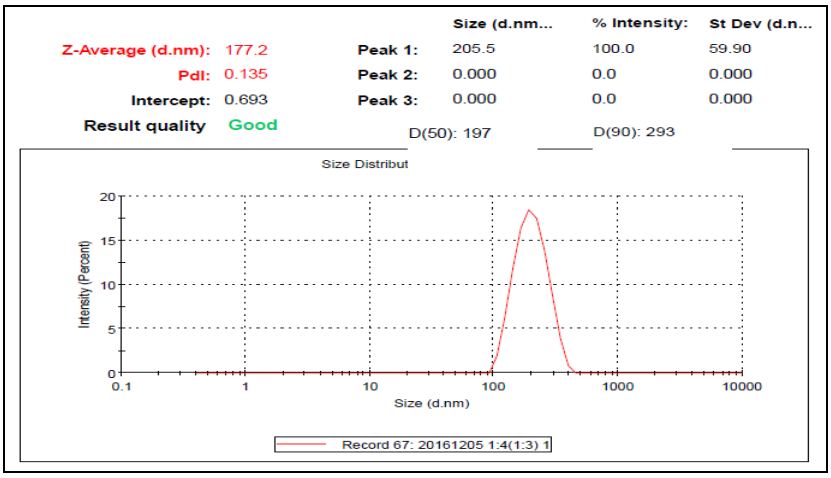

FIG. 2: PARTICLE SIZE DISTRIBUTION OF NE2

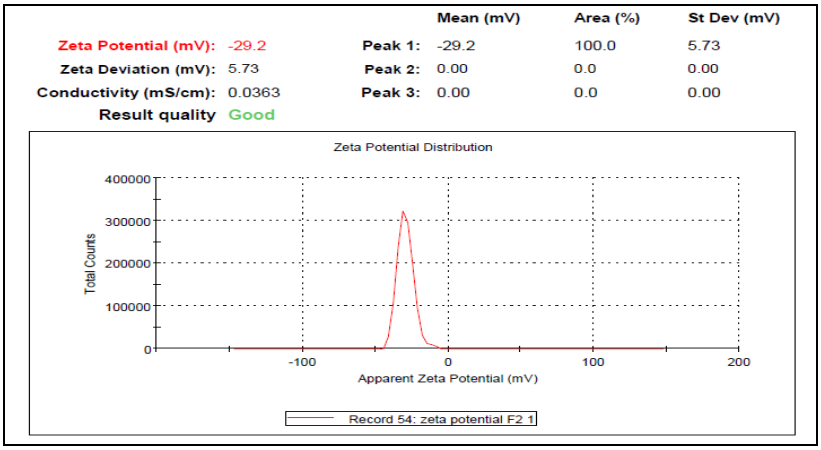

FIG. 3: ZETA POTENTIAL (mV) OF NE2

Viscosity determination: Overall, the viscosity of the optimized formulations was low as noticed in case of for o/w nanoemulsion. NE1 had the minimum viscosity because of higher aqueous content and results were significant as compared to formulations NE2(**Table 3**).

Refractive index: There was no significant change in the mean value of the refractive index of nanoemulsions which were observed even after storage for 1 month (**Table 3**).

Drug Content: NE1 has showed greater amount of drug content as compared to NE2(**Table 3).**

TABLE 3: *IN VITRO* CHARACTERIZATION OF OPTIMIZED NANOEMULSIONS

Nanoemulsion	Droplet size (nm)	Polydispersity Index	Refractive Index	Viscosity (cP)	Drug content (%)
NE1	187.4	0.139	1.352±0.017	18.08±1.14	92.5±0.56
NE2	177.2	0.135	1.354±0.014	20.12±0.95	94.02±0.21

***In vitro* drug release:** Dissolution studies were performed to compare the release of drug from different selected formulations (**Fig. 4**). NE2 was very fast while NE1 showed comparatively slow release. This fact has been attributed that nanoemulsions having comparatively smaller size and hence the larger surface area for dissolution as justified by droplet size distribution. NE2 has revealed (96.88±1.29%) of drug released as compared to NE 1 (85.36±2.12%) in 120 mins suggesting faster release (**Fig. 4**).

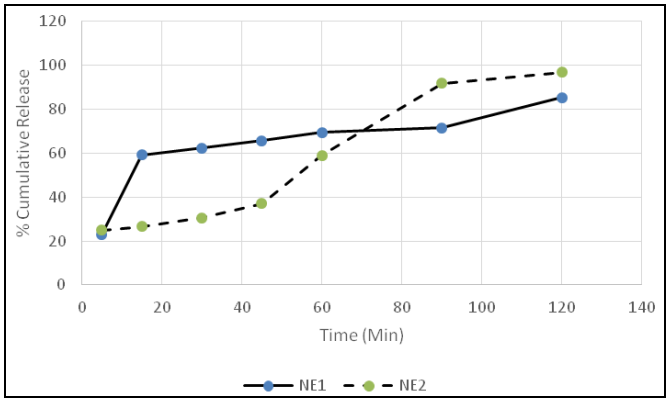

FIG. 4: *IN VITRO* RELEASE PROFILE OF NANOEMULSIONS

Screening of nanoemulsion for *in vitro* anti-inflammatory activity:

Inhibition of albumin denaturation: Denaturation of proteins is a well-documented cause of inflammation [12]. As a part of the investigation on the mechanism of the anti-inflammatory activity, nanoemulsions were studied. They were found to be effective in inhibiting heat induced albumin denaturation at different concentrations as noticed in our results. NE 2 showed maximum inhibition at as compared to NE 1 and Diclofenac Na (**Fig. 5**).

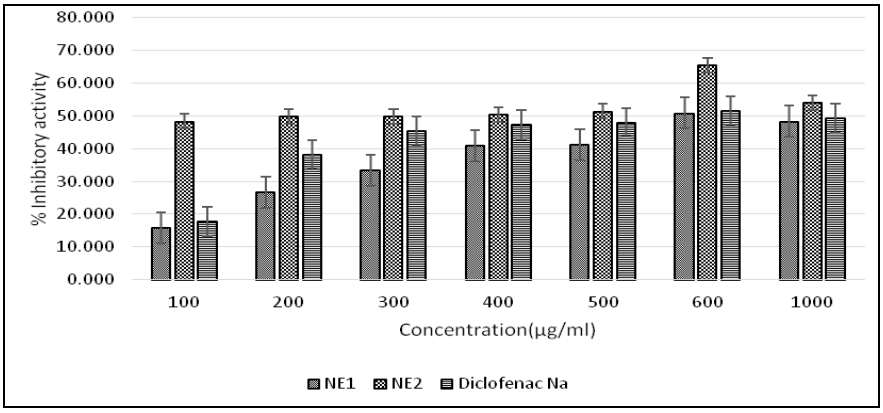

FIG. 5: EFFECT OF NANOEMULSIONS ON PROTEIN DENATURATION

Proteinase inhibitory action: Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a rich source of proteinase which carries in their lysosomal granules many serine proteinases [12]. It has been previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors. Nanoemulsions exhibited significant activity. The nanoemulsions exhibited significant antiproteinase activity at different concentrations as shown in **Fig. 6.**

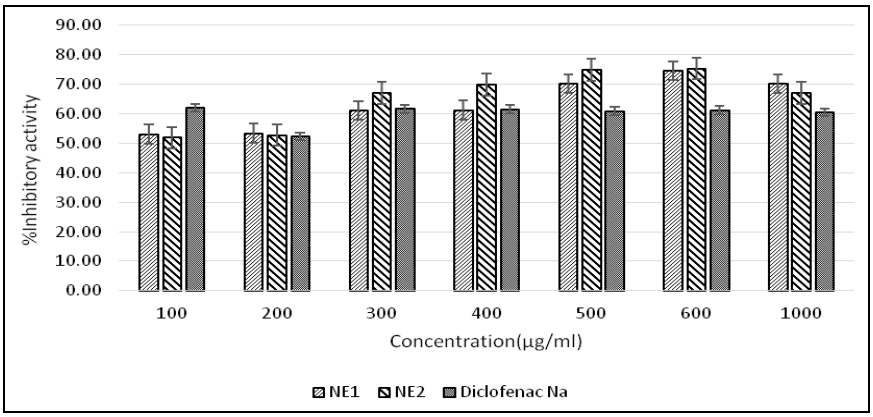

FIG. 6: EFFECT OF NANOEMULSIONSON PROTEINASE INHIBITORY ACTION

Membrane stabilization test: Stabilization of the RBCs membrane was studied to possibly correlate the inhibition of the release of lysosomal content of neutrophils at the site of inflammation. Neutrophil lysosomal constituents include bactericidal enzymes and protease which upon extracellular release cause further tissue inflammation and damage. Nanoemulsions inhibited the heat induced hemolysis of RBCs to varying degree as noticed[12]. Although the precise mechanism of this membrane stabilization is yet to be elucidated, it is possible that the plant developed nanoemulsions could affect surface area/ volume ratio of the cells which could be brought about by an expansion of membrane or the shrinkage of the cells and an interaction with membrane proteins. Nanoemulsions inhibited the heat induced hemolysis of RBCs at 600µg/ml (**Fig. 7**).

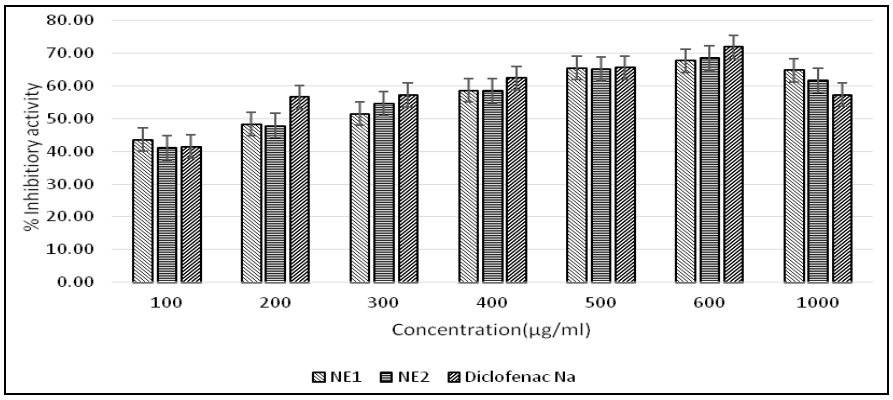

FIG. 7: EFFECT OF NANOEMULSIONS ON MEMBRANE STABILIZATION

Evaluation of nanoemulsions for anti-microbial activity: The NE2 formulation showed excellent antibacterial activity against *Staphylococcus aureus, Bacillus subtilis,* and *Salmonella typhi* whereas NE1 formulation showed excellent antibacterial activity against *Escherichia coli* (**Fig. 8**).

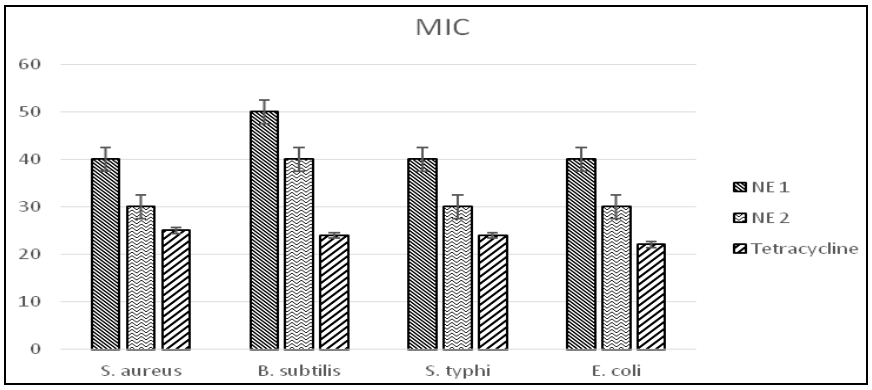

FIG. 8: MINIMUM INHIBITORY CONCENTRATION (MIC)

CONCLUSION: In the present research work, potential of developed nanoemulsion as a drug carrier for oral delivery from petroleum ether plant extracts of *Carissa carandas* (CC) and *Carissa spinarum* (CS) were found to be satisfactory. The anti-inflammatory and antibacterial studies were found to be promising for the developed nanoemulsions.

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CONFLICT OF INTEREST: Authors declare no conflict of Interest.

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