Pharmaceutical Sciences Asia

Pharm Sci Asia 2025; 52(2), 194-205

DOI:10.29090/psa.2025.02.24.2162

Research Article

Targeting Inflammation with *Vitex negundo* Essential Oil Nanoemulsion: *In-Vitro* and *In-Vivo* Investigation

Shanti Bhushan Mishra[1,*], Krishnadhar Dwivedi[2], Amit Kumar Singh[2], Shradhanjali Singh[3]

[1] Department of Pharmacognosy, United Institute of Pharmacy, Prayagraj, India
[2] Department of Pharmaceutics, United Institute of Pharmacy, Prayagraj, India
[3] Department of Pharmaceutical Chemistry, United Institute of Pharmacy, Prayagraj, India

ABSTRACT

Nirgundi oil, an essential natural oil obtained by steam distillation of *Vitex negundo* leaves, exhibits significant biological effects in preventing various types of inflammation and pain. This study aimed to develop Nirgundi oil-loaded nanoemulsions to test their anti-inflammatory effects through *in-vitro* and *in-vivo* models. The nine developed nanoemulsions were assessed for various physicochemical parameters including pH, Fourier Transform Infrared Spectroscopy (FTIR), mean droplet size, polydispersity index (PDI), zeta potential (ZP), and refractive index (RI). The Optimized nanoemulsion(NF5) had a mean droplet size (53.44 ± 0.69 nm), PDI (0.35 ± 0.02), and ZP (–31.9 mV). TEM micrographs of the NF5 nanoemulsion droplets revealed that they were spherical in shape with smooth surfaces. Moreover, the *in- vitro* anti-inflammatory activity of the optimized nanoemulsion (NF5) showed 78.65 ± 1.032 % and 58.48 ± 1.056 % inhibition of hypotonic solution induced hemolysis and heat induced hemolysis respectively at a concentration of 200 µg/ml. Compared with standard and control treatments, NF5 had significant anti-inflammatory activity in carrageenan induced inflammation ($p<0.0001$), and cotton pellet granulomas ($p<0.0001$) in a dose dependent manner. The VNEO nanoemulsion exhibited potent anti-inflammatory activity and could be utilized for managing inflammatory conditions with improved bioavailability.

Keywords:
Anti-inflammatory;Cotton pellet granuloma;Hemolysis; Nanoemulsion; Nirgundi oil; *Vitex Negundo*

1. INTRODUCTION

In recent years, the quest for novel therapeutic agents from natural sources has intensified, driven by the need for safer and more effective treatments for various ailments. The essential oils derived from medicinal plants possess many diverse pharmacological properties including anti-inflammatory activity[1]. Among these, Vitex negundo, commonly known as the five-leaf chaste tree, has been recognized for its traditional use in folk medicine to alleviate inflammation and pain. Vitex negundo essential oil, which is extracted from the leaves of plants, has ample bioactive compounds such as terpenoids, flavonoids, and phenolic compounds, which are attributed to its pharmacological effects[2]. Several researchers have previously reported on the chemical composition of the essential oil found in the leaves of Vitex negundo and other Vitex species[3-4]. This essential oil has been found to possess beneficial properties for treating wounds and ulcers. Moreover, leaves are commonly utilized in the management of various conditions, including inflammation, toothache, rheumatoid arthritis, eye diseases, fever, leucoderma, gonorrhea, ulcers and bronchitis[5-7].

***Corresponding author:**
* Shanti Bhushan Mishra Email: shantibhushanmishra@united.ac.in

Pharmaceutical Sciences Asia © 2024 by
Faculty of Pharmacy, Mahidol University, Thailand is licensed under CC BY-NC-ND 4.0. To view a copy of this license, visit
https:// www.creativecommons.org/licenses/by-nc-nd/4.0/

However, the medicinal application of essential oils is often limited by their hydrophobic nature, volatility, and potential irritant effects. Nanoemulsions, a colloidal dispersions of nanoscale droplets, are a promising solution for enhancing the stability, solubility, and bioavailability of essential oils while preserving their therapeutic efficacy[8].

Nanoemulsions, characterized by their fine droplet size and high stability, offer a platform for improving the solubility, permeability, and targeted delivery of bioactive compounds[9-10]. This research aimed to explore the formulation, optimization, and evaluation of *Vitex negundo* essential oil nanoemulsion as a novel drug delivery system. Furthermore, the study investigated the anti-inflammatory activity of the formulated nanoemulsion, shedding light on its potential pharmacological benefits in managing inflammatory conditions. Through a comprehensive approach encompassing formulation science, optimization techniques, and pharmacological evaluation, this research endeavors to contribute to the development of innovative therapeutic strategies utilizing natural products for the treatment of inflammatory disorders. The findings of this study hold promise for the advancement of healthcare, offering safer and more efficacious alternatives to conventional anti-inflammatory agents.

2. MATERIALS AND METHODS

2.1 Materials

The essential oil of Vitex negundo leaf (Nirgundi essential oil) was purchased from M/S Salvia Cosmeceuticals Pvt. Ltd., New Delhi, India. Carrageenan, Heparin, Tween-80, and Span-80 were obtained from Maddox Biotech India Pvt. Ltd. Chennai, India. The purified water was obtained from Millipore Corporation. Di-sodium hydrogen phosphate, di-hydrogen phosphate, sodium hydroxide, hydrochloric acid, sodium chloride, and ethanol were purchased from Qualigens Pharma, Mumbai, India.All chemicals used in the study were of analytical grade.

2.2 Authentication of *Vitex negundo* essential oil by GC-MS

The Vitex negundo essential oil (VNEO) was authenticated by Gas Chromatography-Mass Spectrometry (GC-MS) to ascertain the presence of bioactive compounds.VNEO was examined using a ShimadzuQP Plus (Shimadzu Corporation, Japan) system through the desorption system including as slit Injector. The following operating conditions were maintained during GC-MS analysis. A capillary column with quartz glass was used and approximately 99% helium gas was used as the carrier gas at a constant running rate of 1.2 ml/min. The injector temperature was 260 ºC, while the ion-source temperatures were 230ºC and 270ºC (1:100). The temperature of the oven was set at 50ºC (isothermal for approximately 2min), followed by amplification at a rate of 3ºC/min to 280ºC, then 10ºC/min to 260ºC, and finally300ºC (for about 10 minutes). For fragments in the range of 40 to 500 Daltons and the mass spectra can be obtained at 70eV with a scan time of 0.5 seconds. Identification of the components of Vitex negundo essential oil was performed by toning the obtained mass spectra data using CSIR library search[11].

Table 1. Composition of various developed nanoemulsions

Formulation Code	Oil: Surfactant Ratio (v/v)	Percent composition of different components in formulation			
		Oil	Surfactant		Water
			Tween-80	Span-80	
NF1	1:1	1	1	1	97
NF2	1:2	1	2	2	95
NF3	1:3	1	3	3	93
NF4	1:4	1	4	4	91
NF5	**1:5**	**1**	**5**	**5**	**89**
NF6	1:6	1	6	6	87
NF7	1:7	1	7	7	85
NF8	1:8	1	8	8	83
NF9	1:9	1	9	9	81

2.3 Preparation of the VNEO nanoemulsion

The nanoemulsions were prepared by previously reported method with slight modification[12]. Initially, coarse emulsions were prepared by mixing the oil phase, aqueous phase and emulsifier using magnetic stirrer at 400rpm for 30 minutes. The oil phase was Nirgundi essential oil, the aqueous phase was distilled water and the emulsifiers used were mixtures of Tween 80 and Span 80 in different proportions as shown in **(Table 1)** The coarse emulsion thus obtained was sonicated for different time interval (15, 30, 45 and

60min) (Probe sonicator USA) to obtain droplet of smaller size and more uniform particle size distribution. The ultrasonic probe tip with a diameter of 13mm served as the energy input device.

2.4 Characterization of the nanoemulsion

2.4.1 pH determination

The pH of the prepared nanoemulsion was determined using a digital pH meter (A & T Scientific Industries) which was previously calibrated using standard buffer solutions at pH 4, 7 and 10. pH was measured by dipping the electrode in nanoemulsion solution without any dilution. All the measurements were carried out in triplicate.

2.4.2 Particle size, PDI and Zeta potential measurement

The particle size, zeta potential and PDI of the developed formulations (NF1-NF9) were determined using a Malvern Zetasizer (Nano ZS90, Malven Instruments Ltd., UK). The samples were diluted with double distilled water (1:100) to avoid multiple scattering.

2.4.3 FTIR spectroscopic analysis

FTIR analysis was conducted on VNEO and VNEO-loaded nanoemulsion (NF5) to identify the functional groups involved and interaction between the above ingredients. The analysis was performed using FTIR (Perkin Elmer spectrum ver. 10.03.02). The resulting spectrum covered a range of 4000 to 400cm-1 with a resolution of 4 cm-1. To prepare the sample KBr pellets were mixed with all the ingredients.

2.4.4 Transmission electron microscopy

The morphological study of droplets in the optimized batch of nanoemulsion was studied using transmission electron microscopy (TEM). The optimized nanoemulsion formulation (NF-5) was stained with 1% phosphotung state. A copper grid with carbon coating was placed on filter paper to absorb any extra nano-dispersion before being coated with one drop of drug-loaded invasomes, which was then allowed to dry to a thin film. This film was colored with 1% phosphotungstatic acid before it was completely dried on the grid. After the sample had dried, it was inspected using a transmission electron microscope with an accelerating voltage of 80 kV. Images were captured at the appropriate magnifications[13]

2.4.5. Drug content determination

The essential oil content in the prepared VNEO loaded nanoemulsion was determined by spectrophotometric method. The nanoemulsion formulations were mixed with n octanol (1:1) diluted in Phosphate Buffer Solution (PBS) pH 7.4 (1:100) and homogenized in ultrasonic bath, after which the amount of essential oil was quantified spectrophotometrically at 263nm[30].

$$\%\ drug\ content = \frac{amount\ of\ drug\ in\ nanoemulsion}{Total\ drug\ incorpoarated} \times 100$$

2.4.6 *In-vitro* Drug Release Study

An in-vitro drug release study of the developed nanoemulsion formulation (NF1-NF9) along with *Vitex negundo* essential oil alone was carried out by the dialysis bag method as previously reported method with minor changes[14]. The dissolution media for the release study was the PBS (pH 7.4). 2 ml of nanoemulsion, 1.5 ml of PBS (pH 7.4) and 0.5 ml of n-octanol were added in dialysis bag-110 (diameter 21.5 mm, flat with 31.13 mm, HI-media, Mumbai, India) and tied at both ends. The dialysis bag was then dipped into a beaker containing release medium and stirred at a speed of 50 rpm and the temperature of the medium was maintained at (37±5ºC). As an effect of diffusion from the membrane, the drug released in the external solution. At intervals of 0, 15, 30, 60, 90, 120, 150, and 180 minutes 5 ml of sample was withdrawn and replaced with fresh media. The samples were analyzed spectrophotometrically at 263nm using UV-spectrophotometer.

2.5 Selection of the optimized formulation

The selection of the optimized formulation for further study was based on the characterization of the nanoemulsion. The formulation exhibiting smallest droplet size, greatest drug release, and an acceptable PDI with good stability was selected as the optimized formulation.

2.6 Pharmacological studies

2.6.1 Animals

Healthy Sprague Dawley Rats (SD Rats) of both sexes aged 2-3 months and weighing150-180 g were procured from CCSEA registered Laboratory Animal Supplier m/s Chakraborty Enterprise Kolkata, W.B., India (Reg.no.1443/PO/Bt/s/11/CPCSEA) and were used for the animal studies. The animals were kept in a departmental animal facility where the

animals were alternately exposed to darkness and light for 12 hours at a temperature of 25±3ºC and 30-60% humidity. The animals were acclimatized to the conditions of animal house for one week prior to the experiments. All the experimental work on animals was approved by the Institutional Animal Ethical Committee of the United Institute of Pharmacy with approval no. UIP/IAEC/Nov.-2021/06.

2.6.2 *In -vitro* anti-inflammatory activity

2.6.2.1 Preparation of Erythrocyte Suspension

The rats were anesthetized during the collection of blood via retro-orbital vein puncture and kept in heparinized vacutainer tubes. The blood was cleansed thrice with 0.9% saline solution and centrifuged at 2500 rpm for 10 minutes. The packed cells were washed with 0.9% saline solution and 40% v/v suspension was prepared using an isotonic phosphate PBS (pH 7.4) which was used as a stock erythrocyte suspension[15]

2.6.2.2 Hypotonic solution induced hemolysis

The experimental procedure was conducted following the methodology outlined by Padmanabhan and Jangle with minor changes[15]. The test sample consisted of a 0.030 ml mixture of stock erythrocyte suspension and 5ml of hypotonic solution (containing 154mM NaCl and 10mM sodium PBS (pH 7.4). Nanoemulsion was added to the hypotonic solution at concentrations ranging from 50, 100 and 200 µg/ml. The control sample contained only a 0.030ml RBC suspension mixed with the hypotonic buffered solution. The standard drug aspirin was subjected to the same treatment as the test at concentration of 200 µg/ml. After the mixtures were incubated for 10 minutes at room temperature, they were then centrifuged at 2500 rpm for 10 minutes. The absorbance of the supernatant was measured using a spectrophotometer at a wavelength of 540 nm.

2.6.2.3 Heat induced hemolysis

The experimental procedure was implemented according to the methodology described by Aidoo et al with minor modifications[16].The experiment involved mixing VNEO-loaded nanoemulsion or aspirin dissolved in isotonic PBS (pH 7.4) with 1 mL of 2% erythrocytes suspension at various concentrations ranging from 50, 100 and 200 µg/ml. The reaction mixture was then incubated at 56 °C for 30 minutes in a water bath. After incubation, the tubes were cooled using running tap water and subsequently centrifuged at 2,000 r/min for 10 minutes. The absorbance of the supernatants was measured at a wavelength of 560 nm. The percentage inhibition of hemolysis was calculated using the following formula.

$$\%\ inhibition = \frac{Absorbance\ (control) - Absorbance\ (test)}{Absorbance\ (control)} X\ 100$$

2.7 Acute oral toxicity of NF5

The acute oral toxicity of the optimized nanoemulsion formulation was evaluated as per Organization for Economic Cooperation and Development (OECD) guideline[17] No. 423. Before acute oral toxicity studies, all the animals were fasted overnight before dosing and for 3 h after dosing. Initially single dose of 50mg/kg was given to, three rats. These animals were examined regularly for clinical signs or mortality periodically during the first 24 hours. When no mortality or obvious signs of toxicity were observed within 24 hours after treatment, the procedures were repeated in an additional three rats using 300 mg/kg of optimized nano-emulsion and were observed for clinical signs or mortality periodically during the first 24 hours as stated earlier. The same procedure was repeated for higher doses of (2000 mg/kg, and 5000 mg/kg). During the toxicity study, all animals were active and no mortality was recorded throughout the experiment hence two doses 100 mg/kg and 200 mg/kg body weight were selected for the in-vivo study.

2.8 *In-vivo* anti-inflammatory activity

2.8.1 Carrageenan-induced rat paw edema

The anti-inflammatory effect of the developed optimized nanoemulsion (NF5) was investigated using carrageenan- induced paw edema in rats according to a previously reported method[18]. The edema was induced by injecting 0.1ml of 1%w/v carrageenan solution into the sub plantar tissue of the right hind paw. The paw volume was measured with a plethysmometer (Basile, Italy) till four hours at interval of one hour. All animals were divided into four groups; each consisting of six rats. Group I served as control group that received orally normal saline solution as a vehicle, Group II served as standard group that received orally standard drug diclofenac sodium (50 mg/kg i.p.), Group III and IV served as test groups that received nanoemulsion (NF5) at a dose of 100 mg/kg and 200 mg/kg body weight respectively through oral route. Inflammation inhibition was calculated using the following formula

$$\%\ inhibition = \frac{(Vt - V0)(control) - (Vt - V0)(treated)}{(Vt - V0)(control)} X\ 100$$

V0: volume measured before administration of carrageenan
Vt: volume measured after the administration of carrageenan

2.8.2 Cotton pellet-induced granuloma

A total of twenty-four SD rats were separated and allocated into four groups, each consisting of six animals. For the experiment, the groin region of the animals was shaved prior to administering anesthesia with light ether. Through a single needle incision, sterile cotton pellets weighing 25±1 mg were inserted into the groin portion. Animals in Group I received normal saline treatment, while those in Group II were given standard diclofenac sodium at a dose of 50 mg/kg intraperitoneally. Groups III and IV received NF5 at doses of 100 mg/kg and 200 mg/kg respectively for seven days from the day of implantation of cotton pellet. The formulation and the standard drugs are administered through oral route. The animals were anesthetized on the 8thday, followed by surgical removal of the cotton pellets and removal of any extraneous tissues. After incubating for 24 hours at 37°C, then the pellets were dried in an oven at 55°C until a constant weight was achieved. The resulting increase in dry weight of the pellets was used as a measure for granuloma formation[19].

2.9 Statistical analysis

The statistical analysis was executed using GraphPad Prism version 9.4.3, windows software by employing Dunnett's test and two-way ANOVA. $P<0.0001$ considered significant as compared with control group.

3. RESULTS AND DISCUSSION

3.1 Gas Chromatography/Mass Spectrometry (GC-MS)

The GC-MS chromatogram **(Figure 1)** revealed several peaks corresponding to different chemical compounds presents in the essential oil. By comparing the mass spectra with reference standards or databases and retention times, the major compounds were identified as delta 3 carene (MW 136; RT 5.74 min), α-pinene (MW 136; RT 6.80 min), nor-pseudoephedrine (MW 151; RT 14.42 min), nor-ephedrine (MW 151; RT 24.31 min) and cis-caryophyllene (MW 204; RT 24.38 min). Common constituents of *Vitex negundo* essential oil include, Monoterpenes: Compounds such as linalool, α-terpineol, and α-pinene are frequently found in *Vitex negundo* essential oil. These monoterpenes contribute to its antimicrobial and anti-inflammatory properties[20-21]. Sesquiterpenes: β-Caryophyllene, caryophyllene oxide, and humulene are examples of sesquiterpene compounds commonly detected in *Vitex negundo* essential oil. Sesquiterpenes often exhibit anti-inflammatory, analgesic, and antioxidant activities[22]. Phenolic compounds: Thymol, carvacrol, and eugenol are phenolic compounds that may be present in *Vitex negundo* essential oil. These compounds possess antimicrobial and antioxidant properties, contributing to the therapeutic effect of the oil[31].

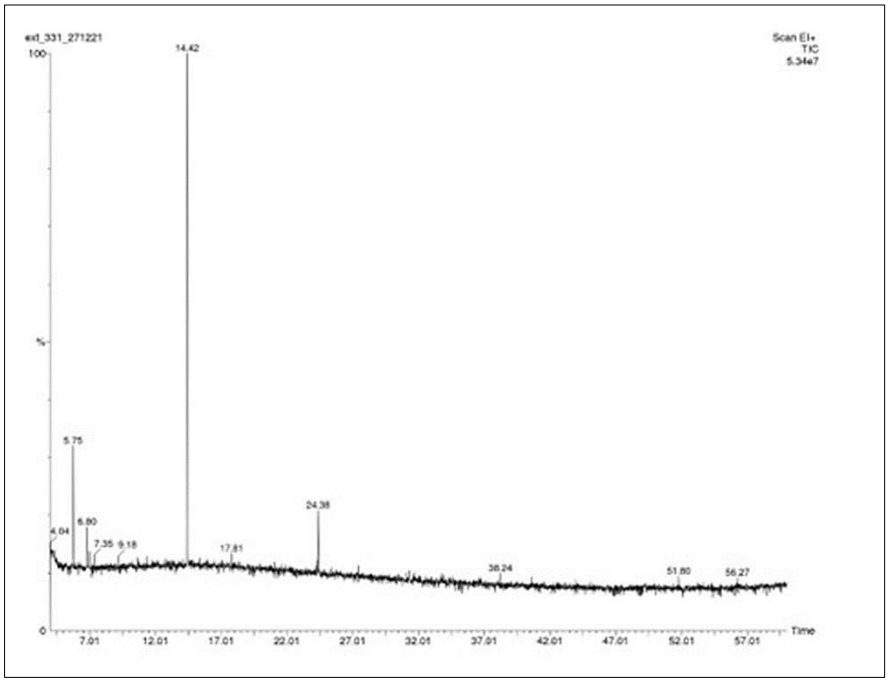

Figure 1. GC-MS spectra of Vitex negundo essential oil (VNEO)

3.2 Nanoemulsion formulation and optimization

The various batches of nanoemulsion formulations NF-1 to NF-9 were formulated as described in the materials and methods section and evaluated. The optimization of the nanoemulsion was carried out by varying the ratio of oil to surfactant concentration and emulsification time (15, 30, 45 and 60min). The results showed that a 60 min emulsification time is sufficient for producing uniform sized nanoemulsion.

3.3 Characterization of nanoemulsion

3.3.1 pH determination

The pH of the optimized nanoemulsion was found to be 5.56 ± 0.63.

3.3.2 Droplet size, PDI, and zeta potential

The droplet sizes of the prepared nano-emulsion were ranged from 53.44±0.69 to 154.09±0.09 nm as shown in Table 2. The lowest droplet size was exhibited by formulation NF5 while the greatest particle size was attributed to formulation NF3. The droplet size of a nanoemulsion is influenced by the ratio of oil to surfactant concentration. The droplet size of the nanoemulsion decreased with increasing surfactant concentration which may be attributed to better stabilization of the droplet with increasing the surfactant concentration subsequently, with increasing surfactant concentration,an increase in droplet size was observed. The PDI value of all formulations were ranged between 0.345±0.09 and 0.412±0.87. In general, the dispersions with PDI value between 0.1 and 0.3 were considered monodisperse particles. The zeta potential is a commonly utilized indicator to predict the charge on element shells and evaluate their stability. The level of system solidity is attributed to the charge present in the nanoemulsion and its tendency to aggregate. Particles are considered repulsive if their zeta potential is at least +32, leading to a separation from each other, while a zeta potential of -30 or lower prevents flocculation and promotes dispersion[23]. The Zeta potential of all formulations ranged from -28.4 to -32.4mV **(Table 2).** The estimated zeta potential for the nanoemulsion (NF5) was found to be -31.9 mV suggesting that the system is evenly spread out and has the potential to maintain stability for an extended duration **(Figure 2).** Transmission electron microscopy (TEM) image of the NF5 formulation, clearly revealed the spherical morphology of the nanoemulsion droplets, and verified the presence of nanometer-sized droplets in the formulated emulsion **(Figure 3).**

Table 2. Particle size and polydispersity index of the prepared batches of nanoemulsion.

Batches	Particle size (nm)	Polydispersity index (PDI)	Zeta Potential (mV)	pH	Drug Content (%)
NF-1	121.08±0.32	0.412±0.87	-28.4	4.46±0.02	46.22±0.81
NF-2	132.67±0.12	0.409±0.43	-28.6	4.52±0.05	51.12±0.11
NF-3	154.09±0.09	0.398±0.08	-29.2	4.67±0.34	54.27±0.13
NF-4	104.02±0.46	0.387±0.56	-30.7	4.87±0.87	58.18±0.81
NF-5	**53.44±0.69**	**0.358±0.02**	**-31.9**	**5.56±0.63**	**82.12±0.32**
NF-6	87.89±0.76	0.376±0.67	-30.8	6.01±0.21	62.24±0.88
NF-7	119.78±0.12	0.369±0.35	-31.2	6.23±0.42	64.82±0.32
NF-8	149.05±0.98	0.358±0.12	-30.3	6.56±0.54	68.11±0.43
NF-9	98.34±0.54	0.345±0.09	-30.1	4.46±0.02	72.19±0.52

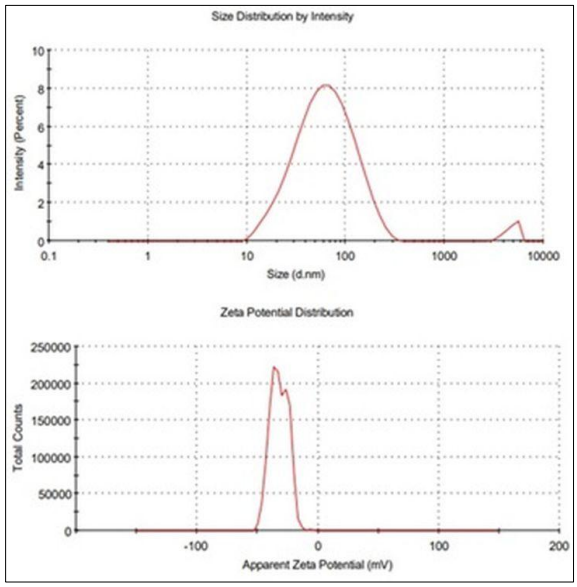

Figure 2. Zeta potential and particle distribution of formulation (NF5)

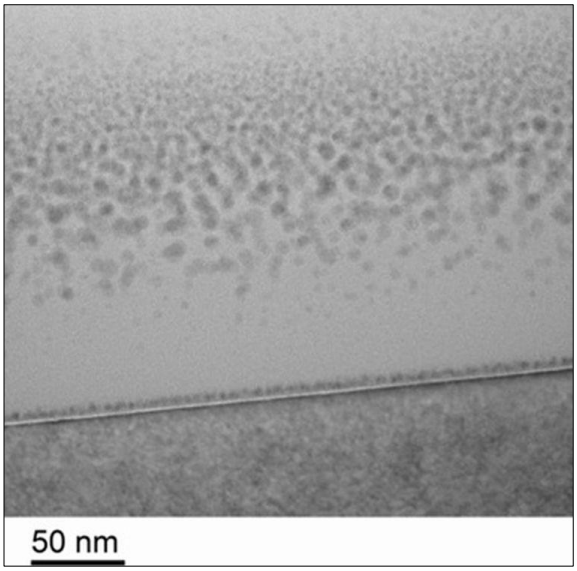

Figure 3. TEM image of optimized formulation (NF5)

3.3.3 FTIR spectroscopic study

The FTIR spectra of VNEO and NF5 are shown in **Figure 4(a-c)**. The variations in wavenumber or fluctuations in peak intensity serve to elucidate the specific functional groups implicated in the binding processes. The FTIR spectrum for VNEO showed the absorption bands of C=O stretching at 1742 cm−1, N-H stretching at 3483.59 cm−1, C-H stretching at 2823.36 cm−1 and 2857.20 cm−1, C-O stretching at 1159.61 cm−1, C-I stretching at 592.61 and CH_2bending at 1452.92 cm−1 were obtained Figure 4 (a). FTIR spectra of prepared NF5 showed N-H stretch shifted to 3344.17 cm−1, C=C(aromatic) stretch shifted to 1638.37 cm−1 and 2117.88 cm−1, C-H stretch at 2938.8cm−1 and C-I stretch at 599.24 cm−1 Figure 4 (c). The presence of carbonyl groups in VNEO and NF5 confirmed the presence of flavonoids or terpenoids[24]. The FTIR spectrum exhibits some shifting of the peaks which suggests that the functional groups are responsible for the binding mechanism of NF5.

The FTIR results indicate that no chemical interactions occurred during the formulation of NF5 which is compatible with other excipients Figure 4 (b). The chemical makeup of these monoterpenes and sesquiterpenes varies, ranging from a simple carbon and hydrogen molecules to more complicated compounds such as alcohols, aldehydes, ketones, and

ethers that have oxygenated organic groups. Numerous compounds containing 10 or 15 carbon atoms exhibit significant biological activity. Prior research[25-26] indicates that the presence of terpenes, such as alpha pinene and β-Caryophyllene validates their effectiveness as anti-inflammatory agents.

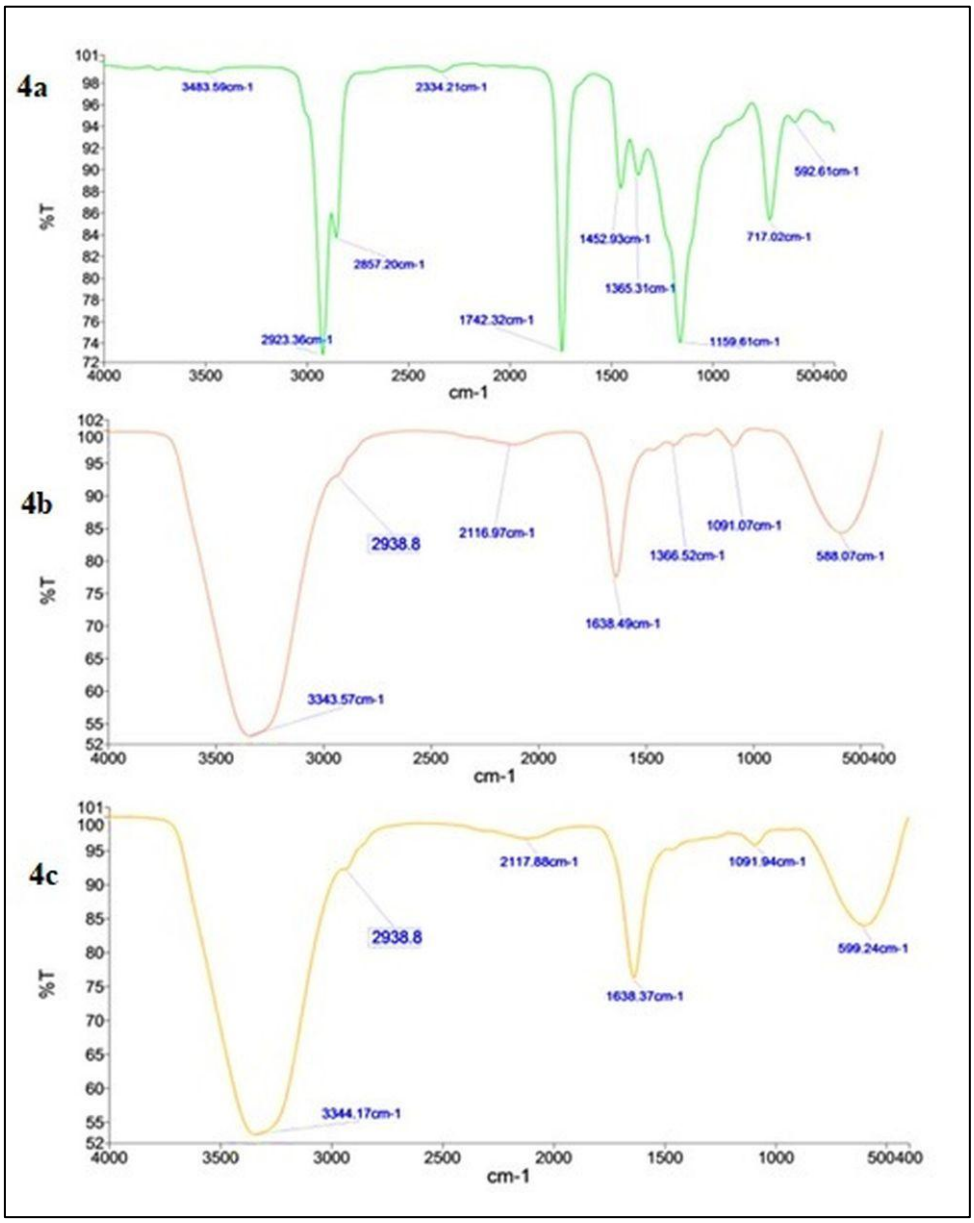

Figure 4. FTIR spectra of VNEO (4a), Excipients (4b) and NF5 (4c)

3.3.4 Drug content

The drug content of NF-5 reached a maximum of approximately 82.12µg/ml.

3.3.5 Drug release study

PBS (pH 7.4) was used for in vitro drug release testing of VNEO and the nanoemulsion. The results showed that VNEO had a maximum in vitro drug release of 38.9% within 24 hours. In comparison, the NF1-NF9 formulations of VNEO-loaded nanoemulsions exhibited drug release rates between 48.02% and 93.43% within the same time period. Notably, NF5 displayed the highest dissolution rate with a maximum drug release of 93.43% within 24 hours. This is likely due to its smaller droplet size, which provided a larger surface area for drug release **(Figure 5).**

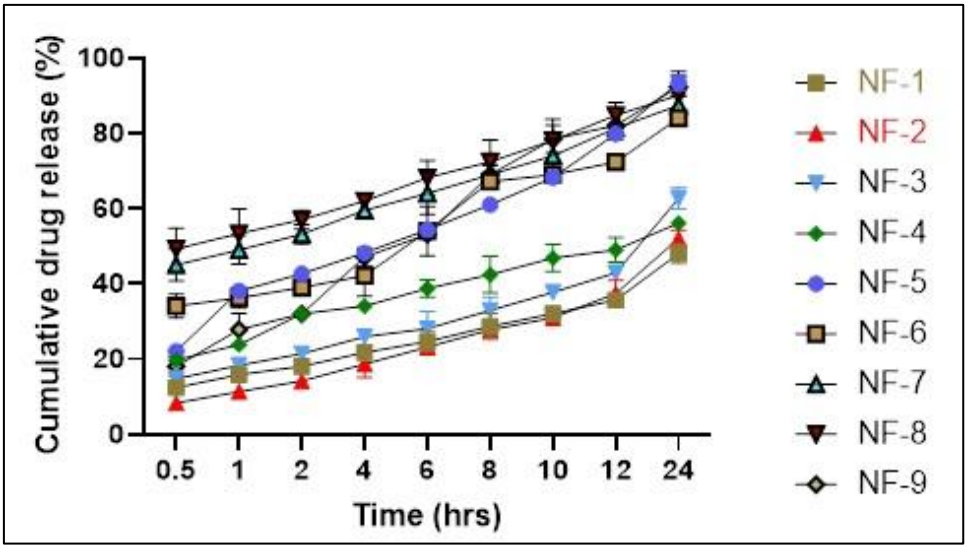

Figure 5. Cumulative drug release of prepared nanoemulsions (NF1-NF9)

3.4 In vitro anti-inflammatory activity

The findings of the in vitro anti-inflammatory activity revealed that NF5 had a concentration-dependent anti-inflammatory effect and protected the erythrocyte membrane from exposure to the hypotonic solution and heat. The maximum percentage hemolysis inhibition was found to be 78.65 ± 1.03% at a concentration of 200 µg/ml in the hypotonic solution induced hemolysis whereas at same concentration of NF5, protected the erythrocytes from heat and shows percent inhibition about 58.48±1.05%. At the same concentration, the standard drug aspirin inhibited hemolysis by approximately82.34±1.54% in hypotonic solution induced hemolysis and 23.09±1.86% in heat induced hemolysis. **(Figure 6).**

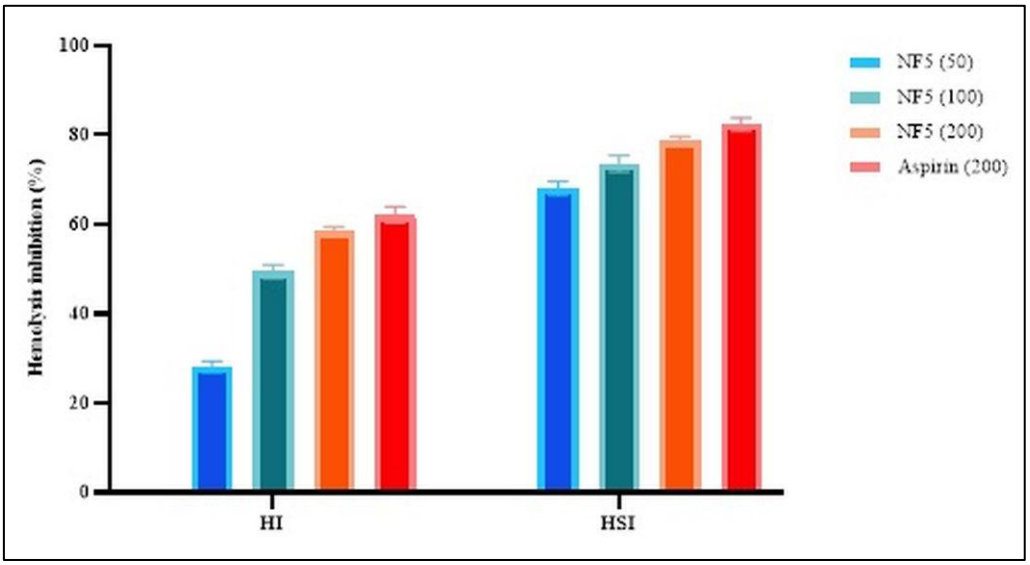

Figure 6. Effect of NF5 on Heat induced (HI) and Hypotonic solution induced (HSI) hemolysis

The erythrocyte membrane is widely recognized as a structurally similar to the lysosomal membrane. The presence of a stable erythrocyte membrane suggests the potential for lysosomal membrane stabilization. Our study revealed that NF5 significantly preserved the erythrocyte membrane from both hypotonic solution and heat-induced lysis. Hemolysis induced by hypotonicity is caused by an excess accumulation of fluid in red blood cells, resulting in rupture of the cell's membrane. In addition, exposing the erythrocyte membrane to intense heat can lead to membrane lysis and oxidation of its haemoglobin[27]. Therefore, stabilizing the erythrocyte membranes can prevent rupture and the release of activated neutrophil cytoplasmic components, such as proteases and bactericidal enzymes, which can further worsen the inflammatory response when released outside the cell[28]. Our results

suggest that the ability of NF5 to stabilize cell membranes might be due to the increase in the surface area of the cells.

3.5. *In- vivo* anti-inflammatory activity

3.5.1. Carrageenan-induced rat paw edema

In the acute inflammation model induced by carrageenan, a significant decrease in paw volume was observed in animals treated with NF5 at doses of 100 mg/kg and 200 mg/kg. This was evident when compared to the disease control group ($P<0.01$) and the standard group treated with diclofenac sodium at a dose of 50 mg/kg. The potency of NF5 at a dose of 200 mg/kg was greater than that at a dose of 100 mg/kg **(Figure 7)**. The intraplantarinjection of carrageenan causes paw edema due to the release of mediators like histamine, serotonin, and kinins that can lead to increased vascular permeability, neutrophil accumulation in inflamed tissue and increased production of oxygen-derived free radicals, prostaglandins and inducible cyclooxygenase[29]. However, oral administration of the NF5 suppressed the oedematous response after 3h and this effect was compared with standard drug diclofenac sodium. These findings suggest that nanoemulsion has anidentical effect to non-steroidal type anti-inflammatory drugs possibly due to the inhibition of prostaglandin biosynthesis or accumulation of neutrophil in inflamed site.

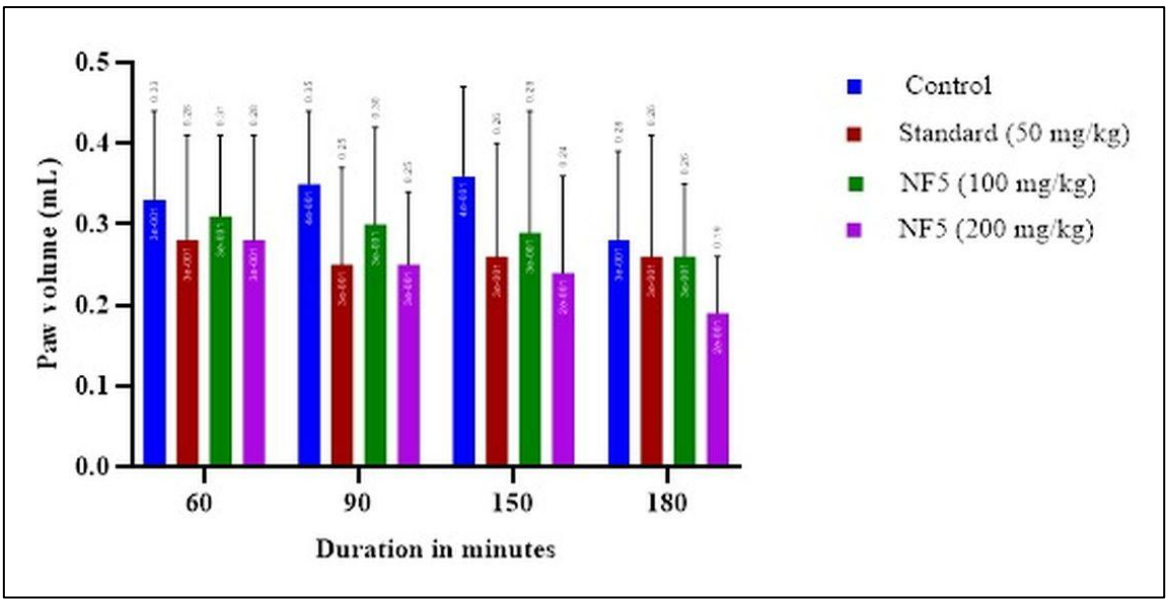

Figure 7. Effect of NF5 oncarrageenan induced paw oedema

3.5.2 Cotton pellet induced granuloma

In this study, the optimized nanoemulsion NF5 was administered in two different doses and evaluated for its impact on granuloma dry weight. The objective of this assessment was to examine the effect of NF5 on the proliferative phase of inflammation. In cotton pellet induced granuloma model, optimized nano-formulation (NF5) at doses of 100 and 200 mg/kg as well as standard drug diclofenac sodium treatment were found to have a significant ($P<0.001$) impact in decreasing granuloma formation. The cotton pellet granuloma method is a commonly used approach for evaluating the transudative, exudative, and proliferative stages of subacute inflammation[30]. Our results demonstrate that NF5 showed effectiveness in inhibiting the formation of granulomas in a dose-dependent manner **(Figure 8).**

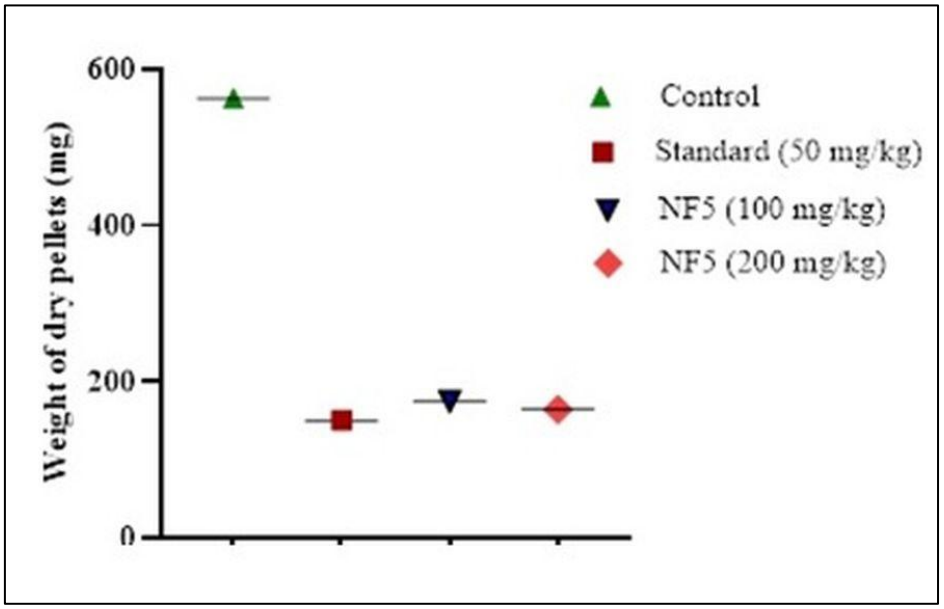

Figure 8. Effect of NF5onCotton pellet induced granuloma

4. CONCLUSION

As noted from above-mentioned investigations, it can be concluded that nanoemulsions containing *Vitex negundo* essential oil were successfully developed with a size of approximately 50 nm using a combination of Tween 80 and Span 80. The optimized nanoemulsion (NF5) demonstrated remarkable anti-inflammatory properties in rats. These findings suggest that the process of nano-emulsification enhances the pharmacological efficacy of VNEO, thereby decreasing the necessary dosage for achieving oral anti-inflammatory effects without any observable toxic consequences. Therefore, the utilization of VNEO loaded nanoemulsion has the potential to enhance bioavailability and improve patient compliance in the treatment of acute and subacute inflammation.

5. ACKNOWLEDGMENT

The authors thank Dr. Alok Mukerjee, Principal, United Institute of Pharmacy, for providing access to the animal house facility to conduct our experiments on animals. We extend our sincere thanks to the Sophisticated Analytical Instrument Facility, Central Drug Research Institute Lucknow for providing GC-MS and FTIR results.

Author contribution

SBM: Conceptualization, Conceived and design the experiments. Analyzed and interpreted the data, writing original draft.

KD: Performed experiments, analyzed and interpreted the data

AKS: Writing, review and editing

SS: Perform experiments, analyzed and interpreted the data.

Conflict of interest statement

None to declare

Funding

None to declare

Ethics approval

Authors have followed all applicable international, national, and/or institutional guidelines for the care and use of animals. The animal studies were accomplished according to CCSEA guidelines. The animal studies were approved (Approval no. UIP/IAEC/MARCH/2023-07) by IAEC of United Institute of Pharmacy, Prayagraj, India.

Article info:

Received May 2, 2024

Received in revised September 10, 2024

Accepted October 4, 2024

REFERENCES

1. Chaachouay N, Zidane L. Plant-Derived Natural Products: A Source for Drug Discovery and Development. Drugs and Drug Candidates 2024; 3(1): 184-207.
2. Kamal N, Mio Asni NS, Rozlan INA, Mohd Azmi MAH, Mazlan NW, Mediani A, et al. Traditional medicinal uses,

phytochemistry, biological properties, and health applications of Vitex sp. Plants 2022; 11(15): 1944.
3. Basu N, Singh G.A Note on the Chemical Investigation of *Vitex egundo* L. The Ind J Pharm.1944; 6: 71-4.
4. Taneja S, Gupta R, Dhar K, Atak C. The essential oil of *Vitex negundo*. Ind Perf.1979; 23: 162.
5. Rameshwar D, Virendra S. A comparative study of volatile constituents of *Vitex negundo* leaves. J Med Arom Plant Sci.2000; 22(1B): 639-40.
6. Khokra S, Prakash O, Jain S, Aneja K, Dhingra Y. Essential oil composition and antibacterial studies of *Vitex negundo* Linn. extracts. Ind J Pharm Sci.2008; 70(4): 522.
7. Huang HC, Chang TY, Chang LZ, Wang HF, Yih KH, Hsieh WY, et al. Inhibition of melanogenesis versus antioxidant properties of essential oil extracted from leaves of *Vitex negundo* Linn and chemical composition analysis by GC-MS. Molecules2012; 17(4): 3902-16.
8. Liao W, Badri W, Dumas E, Ghnimi S, Elaissari A, Saurel R, et al. Nanoencapsulation of essential oils as natural food antimicrobial agents: An overview. Appl Sci.2021; 11(13): 5778.
9. Shaker DS, Ishak RA, Ghoneim A, ElhuoniMA. Nanoemulsion: A review on mechanisms for the transdermal delivery of hydrophobic and hydrophilic drugs. Scientia Pharm.2019; 87(3): 17.
10. Sambhakar S, Malik R, Bhatia S, Al Harrasi A, Rani C, Saharan R, et al. Nanoemulsion: an emerging novel technology for improving the bioavailability of drugs. Scientifica. 2023; 2023:6640103.
11. Borges RS, Keita H, Ortiz BLS, dos Santos Sampaio TI, Ferreira IM, Lima ES, et al. Anti-inflammatory activity of nanoemulsions of essential oil from Rosmarinus officinalis L.: in vitro and in zebrafish studies. Inflammopharmacol.2018; 26(4): 1057-80
12. Song R, Lin Y, Li Z. Ultrasonic-assisted preparation of eucalyptus oil nanoemulsion: Process optimization, in vitro digestive stability, and anti-Escherichia coli activity. UltrasonSonochem. 2022; 82:105904.
13. Touitou E, Dayan N, Bergelson L, Godin B, Eliaz M. Ethosomes - novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. J Control Release. 2000; 65(3):403-18.
14. Séguy L, Groo AC, Goux D, Hennequin D, Malzert-Fréon A. Design of Non-Haemolytic Nanoemulsions for Intravenous Administration of Hydrophobic APIs. Pharmaceutics. 2020; 12(12):1141.
15. PadmanabhanP, Jangle S. Evaluation of in-vitro anti-inflammatory activity of herbal preparation, a combination of four medicinal plants. Int J Basic Applied Med Sci.2012; 2(1): 109-16.
16. Aidoo DB, Konja D, Henneh IT, Ekor M. Protective Effect of Bergapten against Human Erythrocyte Hemolysis and Protein Denaturation *In Vitro*. Int J Inflam. 2021; 2021:1279359.
17. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Test No. 423: Acute Oral toxicity - Acute Toxic Class Method, 2001.
18. Amdekar S, Roy P, Singh V, Kumar A, Singh R, Sharma P. Anti-inflammatory activity of lactobacillus on carrageenan-induced paw edema in male wistar rats. Int J Inflam. 2012; 2012:752015.
19. de Cássia da Silveira e Sá R, Andrade LN, de Sousa DP. A review on anti-inflammatory activity of monoterpenes. Molecules. 2013; 18(1):1227-54.
20. Zareshahrabadi Z, Saharkhiz MJ, Izadpanah M, Iraji A, Emaminia M, Motealeh M, Khodadadi H, Zomorodian K. Chemical Composition and Antifungal and Antibiofilm Effects of *Vitex pseudo-negundo* Essential Oil against Pathogenic Fungal Strains. Evid Based Complement Alternat Med. 2023; 2023:3423440.
21. Di Sotto A, Mancinelli R, Gullì M, Eufemi M, Mammola CL, Mazzanti G, Di Giacomo S. Chemopreventive Potential of Caryophyllane Sesquiterpenes: An Overview of Preliminary Evidence. Cancers (Basel). 2020; 12(10):3034.
22. Kurpiers M, Wolf JD, Steinbring C, ZaichikS, Bernkop-SchnürchA. Zeta potential changing nanoemulsions based on phosphate moiety cleavage of a PEGylated surfactant. JMoleLiquids2020; 316: 113868.
23. Cayuela-Sánchez JA, El Ouaddari A, El Amrani A, Jamal-Eddine J. Rapid determination of essential oils functional groups using compositional methods and VisNIR spectroscopy. J Pharm Biomed Anal. 2023; 227:115278.
24. Nandiyanto ABD, OktianiR, RagadhitaR. How to read and interpret FTIR spectroscope of organic material. Indonesian JSci Technol.2019; 4(1): 97-118.
25. Agatonovic-Kustrin S, Ristivojevic P, Gegechkori V, Litvinova TM, Morton DW. Essential oil quality and purity evaluation via ft-ir spectroscopy and pattern recognition techniques. Appl Sci.2020; 10(20):7294.
26. Ullah HM, Zaman S, Juhara F, Akter L, Tareq SM, Masum EH, Bhattacharjee R. Evaluation of antinociceptive, in-vivo & in-vitro anti-inflammatory activity of ethanolic extract of Curcuma zedoaria rhizome. BMC Complement Altern Med. 2014; 14:346.
27. Kumar V, Bhat Z, Kumar D, Bohra P, Sheela S. In-vitro anti-inflammatory activity of leaf extracts of Basella alba linn. Var. alba. Int J Drug Dev Res.2011; 3(2): 176-9.
28. Ashok P, Koti BC, Thippeswamy AH, Tikare VP, Dabadi P, Viswanathaswamy AH. Evaluation of Antiinflammatory Activity of Centratherum anthelminticum (L) Kuntze Seed. Indian J Pharm Sci. 2010; 72(6):697-703.
29. Hisamuddin N, Shaik Mossadeq WM, Sulaiman MR, Abas F, Leong SW, Kamarudin N, et al. Anti-Edematogenic and Anti-Granuloma Activity of a Synthetic Curcuminoid Analog, 5-(3,4-Dihydroxyphenyl)-3-hydroxy-1-(2-hydroxyphenyl) penta-2,4-dien-1-one, in Mouse Models of Inflammation. Molecules. 2019; 24(14):2614.
30. Mishra SB, Singh D, Singh AK, Singh S. Encapsulation of Thyme Oil into Microsponges: Preparation, Characterization and In Vitro Evaluation. Indian Drugs. 2023; 60(6): 76-82. https://doi.org/10.53879/id.60.06.13428.
31. Khokra SL, Prakash O, Jain S, Aneja KR, Dhingra Y. Essential Oil Composition and Antibacterial Studies of Vitex negundo Linn. Extracts. *Indian J Pharm Sci*. 2008;70(4):522-526. doi:10.4103/0250-474X.44610.