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Research Article

A COMPARITIVE STUDY OF ANTHELMINTIC ACTIVITY OF ALBENDAZOLE NANO EMULSION CONTAINING OREGANO ESSENTIAL OIL

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ABSTRACT

Aim of this work was a comparative study of anthelmintic activity of albendazole nanoemulsion containing oregano essential oil with a marketed formulation.. A problem stated with the use of synthetic anthelmintics is the development of parasitic resistance, which threaten the success of treatment in humans, use of a herbal alternative may reduce such resistance .Essential oil of *Oreganum vulgare* when taken orally kills intestinal parasites, therefore it can enhance the action potential of anthelmintics. Nanoemulsion containing drug and oregano essential oil (NEO) was prepared. And its various evaluation studies were done. The formulated product and marketed product was then evaluated in artificial laboratory conditions by using the earthworms (*Lumbricusterrestris*). Various parameters such as mean paralytic time, mean death time, drug content, invitro drug release studies of both were done. The formulate nanoemulsion had shown a highly better action than that of the marketed product. This study suggest that nanoemulsion is a promising novel formulation that can enhance the solubility of poorly soluble drug like albendazole and thereby enhance its oral bioavailability.

INTRODUCTION

Nanotechnology comprises technological developments on the nanometer scale, usually 0.1-100nm.The use of nanotechnology in pharmaceuticals and medicine has grown over the last few years. The pharmaceuticals developed on the basis of nanotechnology are termed as nanopharmaceuticals. Nanoemulsions are defined as isotropic, thermodynamically stable, transparent or translucent dispersions of oil and water stabilized by an interfacial film of surfactant molecules having the droplet size 20-500nm[1].

Major components of nanoemulsion

Oils

Selection of an appropriate oily phase is very important as it influences the selection of other ingredients of nanoemulsions, mainly in case of O/W nanoemulsions[1]. Usually, the oil which has maximum solubilising potential for selected drug candidate is selected as an oily phase for the formulation of nanoemulsions. This helps to achieve maximum drug loading in the nanoemulsions. The choice of oily phase is often a compromise between its ability to solubilize the drugs and its ability to facilitate formation of nanoemulsion of desired characteristics. Thus mixture of oils can be used to meet both the requirements. For example, a mixture of fixed oil and medium chain triglycerides is used to have good balance between drug loading and emulsification. Apart from the basic component of the nanoemulsion, oregano essential oil is also added, as it has anthelmintic property due to the presence of carvacrol and thymol. Which increases the cytoplasmic permeability of H^+ and K^+ ions in the worms, and also inhibit the production of ATP, causing the death[2].

Cosurfactants

Cosurfactants penetrate into the surfactant monolayer providing additional fluidity to interfacial film and thus disrupting the liquid crystalline phases which are formed when surfactant film is too rigid. Usually a very low HLB cosurfactant is used with a high HLB surfactant to modify the overall HLB of the system. Unlike surfactant, the cosurfactant may not be capable of forming self-associated structures like micelles on its own. Hydrophilic cosurfactants preferably alcohols of intermediate chain length such as hexanol, pentanol and octanol, which are known to reduce the oil/water interface and allow the spontaneous formation of nanoemulsion[2].

Organic solvents such as ethanol, glycerol, propylene glycol (PG), polyethylene glycol(PEG) are suitable for oral delivery, and they enable dissolution of large quantity of either the hydrophilic surfactant or the drug in the lipid base by co-

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solvency and by making the environment more hydrophobic by reducing the dielectric constant of water.

Aqueous Phase

The droplet size and stability of nanoemulsion is influenced by the nature of aqueous phase. Hence, pH and ionic content of aqueous phase should be given due importance while designing. nanoemulsion. The physiological milieu has diverse pH ranges varying from pH 1.2 (pH in stomach) to 7.4 and greater (pH of blood and intestine). In addition, the presence of various ions in the physiological milieu can also have considerable effect on the properties of nanoemulsions[3].

Methods of formulation

1. High-Pressure Homogenisation
2. Microfluidization
3. Spontaneous Emulsification

Advantages of nanoemulsions

- Conversion into nanoemulsion is the approach to improve water solubility and ultimate bioavailability of lipophilic drugs. The nano-sized droplets, with enormous interfacial areas would influence the transport properties of the drug, an important factor in sustained and targeted drug delivery.
- Nanoemulsions have a higher solubilization capacity than simple micellar solutions.Their thermodynamic stability offers advantages over unstable dispersions like emulsions and suspensions because they can be manufactured with little energy input (heat or mixing) and have a long shelf life.
- Nanoemulsions have make the plasma concentration profiles and bioavailability of drugs more reproducible.
- Nanoemulsions have potential to deliver peptides that are prone to enzymatic hydrolysis in GIT[5].

Disadvantages of nanoemulsions

1. Large concentration of surfactants /cosurfactants is required for stabilization.
2. Its stability is affected by temperature .
3. Instability can be caused due to Oswald ripening effect[5].

Anthelmintics

The term anthelmintic is restricted to drugs acting locally to expel parasites from gastro intestinal tract. There are several types of worms which penetrate other tissues, drugs which act on these parasitic infections are also known as anthelmintics.

Albendazole

Albendazole is a benzimidazole useful in the treatment of intestinal nematode infection and echinococcosis[16]. It is effective against roundworm, hookworm, whipworm and threadworm infestations. It is effective in the treatment of ascariasis.

Mechanism of Action

Benzimidazoles selectively bind to nematode ß-tubulin, inhibiting polymerization, thus preventing the formation of microtubules and so stopping cell division. Impaired uptakeof glucose, leading to depletion of glycogen, and reduced stores of ATP has also beennoted.

Pharmacokinetics

Albendazole is poorly absorbed. The parent drug is undetectable in human plasma when 400 mg is taken orally. A single 400 mg dose of the parent drug yields a peak plasmaconcentration of Albendazole in the range 0.22-0.25 mg/L two to three hours post-dose. The estimated terminal half- life of Albendazole is 8.5 hours.

Dosage

A single dose of 400 mg is recommended for clearance of gastrointestinal nematode infection of both adults and children over 2 years of age[6].

MATERIALS AND METHODS

Albendazole and triacetin was purchased from Balaji Chemicals Mumbai, oregano essential oil from chrysalis essentials, Noida. All the other chemicals used were of analytical and laboratory standards.

Characterisation of Albendazole

Solubility

100 mg of drug was taken in a test tube containing 10 ml 7.4 phosphate buffer containing 1%w/v Tween 80. Beaker was shaken occasionally for 24 h and maintained at 25°C. The solution was centrifuged and supernatant filtered through No.1 Whatmann filter paper. Diluted suitably and analyzed by UV spectrophotometer (V-630, Jasco) at 2314nm.

Melting point

Melting point of Albendazole was determined using melting point apparatus (Optic technology). The drug sample was taken in a small capillary tube and placed in the melting point determination apparatus. The melting point of the sample was examined visually through the window and the sample melted temperature was noted.

UV-Vis. Spectroscopy

The UV spectrum of Albendazole was taken in distilled water and methanol (1:1), in a JASCO V-630 spectrophotometer over a wavelength range of 200-400 nm.

FT-IR spectroscopy

FT-IR spectrum of Albendazole was obtained. The sample was made into pellets with KBr and FT-IR model Thermo Nicolet, Avatar 370was used for obtaining spectra. The spectrum obtained was compared with reported data of Albendazole.

XRPD

XRPD pattern of Albendazole was obtained. XRPD was performed at room temperature with Philip's X'Pert Pro X-ray diffractometer (Almelo, Netherlands), voltage 40 kV. The diffraction pattern was recorded from 10 to 60° at an angle 2ɵ and compared with reported data.

Characterisation of Triacetin

Solubility

Solubility of triacetin was determined qualitatively in various media.

Viscosity

Viscosity of triacetin was determined using Brookfield rheometer viscometer RVDVE at 30°C with a CPE 01 spindle at 30 rpm.

FT-IR spectroscopy

FT-IR spectrum of triacetin was taken and compared with reference spectrum for confirmation. FT-IR modelThermo Nicolet, Avatar 370was used for obtaining spectra. The spectrum obtained was compared with reported data of triacetin[7].

Characterisation of Oregano Essential Oil

Solubility

Solubility of oregano essential oil was determined qualitatively in various media.

Viscosity

Viscosity of Triacetin was determined using Brookfield rheometer viscometer RVDVE at 30°C with a CPE 01 spindle at 30 rpm.

GC-MS

GC-MS spectrum of oregano essential oil was taken using GC-MS model Varian 1200 L Single Quadrupole and Fragment ion peaks were obtained. The fragment ion peak m/z obtained was compared with m/z ratio of the standard.

Drug-Oil Compatability Studies

Prior to formulation, to study the chemical compatability of drug with oil phase triacetin and Oregano essential oil were determined, the following studies were conducted on drug and oil mixtures. Ratio of drug and oil(1:1).

FT-IR spectroscopy

The FT-IR spectrum of drug-oil mixture was obtained. FT-IR model Thermo Nicolet, Avatar 370was used for obtaining spectra. The spectrum obtained was compared with reported data of drug and with spectra of oil.

GC-MS

GC-MS spectrum for drug-oil mixture was taken using GC-MS model Varian 1200 L Single Quadrupoleand Fragment ion peaks were obtained. The fragment ion peak obtained was compared with m/z ratio of the individual components.

Formulation - Design and Development

Pseudo ternary phase diagram

Based on the partition coefficient value of essential oil oregano oil was selected. Triacetin was selected as the oil phase. Tween 80 was used as the surfactant. The Co-Surfactant Ethanol was selected. The ratio of surfactant to cosurfactant was fixed at different ratios 1:1,2:1,3:1,4:1,5:1 on the volume basis for each phase diagram. The mixture of surfactant to cosurfactant is refered to as smix in the following discussion. The oil phase was mixed with smix phase in different ratio. An o/w microemulsion technique was employed for the preparation of pseudo ternary phase diagrams. The diagrams were created using Design- expert software. Distilled water was added drop by drop to the mixture of oil and smix after each water addition, the mixture stirred by using vortex mixer until homogenous solution was obtained. The end point was the appearance of turbidity. The quantity of water required to make the mixture turbid was noted. In the Pseudo ternary phase diagram each axis represents aqueous phase, Oil phase and Smix respectively[38]. The area in each of this phase diagram corresponds to the critical points. The criteria of choosing respective ratio depends on the area and desirability value of graphs. The critical point was the point at were the turbidity was observed. Five phase diagrams were constructed taking ratios 1:1, 1:2 ,1:3, 1:4, 1:5[8].

Preparation of nanoemulsion by spontaneous emulsification method followed by Ultrasonication

From the pseudo ternary phase diagram, the formulation showing clear nanoemulsion was selected. The formulation was previously analysed at different oil/smix ratios for optimisation. The formulation composition for the respective nanoemulsion was obtained. 1% of oregano essential oil was mixed with Smix (Triacetin and Ethanol).Required quantity of water was added in drop wise manner and mixed using a magnetic stirrer, until transparent emulsion was formed. The emulsion obtained was then ultrasonicated using Ultrasonic bath sonicator 1.5 L(H Pcianalyte) for 60 minutes. For the incorporation of drug ,it was dissolved in mixture of oregano essential oil and smix and mixed using magnetic stirrer for 15 minutes until a homogenous solution is obtained followed by the above procedure[9].

Formulation of Nanoemulsion

Table 1 Formulation of nanoemulsion (NEO)

Formulation Code	NEO
Triacetin %V/V IN 10 ml	1
Smix %V/V(TWEEN 80 AND Ethanol) IN 10 ml	3
WATER%V/V	5
Amount of Drug Added mg	100
Oil:Smix	1:3
Smix Ratio	1:1
Oregano Oil %V/V IN 10 ml	0.1

The prepared nanoemulsion were subjected to various studies

Anthelmintic Activity

Preparation of Drug Solutions

Albendazole was purchased from Balaji chemicals, Mumbai. The formulation NEO was assessed for its anthelmintic activity. Albendazole 100mg/ml in ethanol was used as Standard and ethanol was used as control.

Collection of Earthworms

Earthworms (*Pheretimaposthuma*) were collected from Agricultural college, Vellayani. The assay was performed *in vitro* using adult earthworms owing to their anatomical and physiological resemblance with the intestinal round worms and parasites of human beings for preliminary evaluation of anthelmintic activity.

Evaluation of Anthelmintic Activity Using Earthworms

Earthworms six number, each of average length of 6 cm, were placed in Petri dishes containing the formulations NEO and Albendazole (Standard).This was done after pouring the Petri

dishes content in to a flat surface and allowing the worms to move freely. Motility of the worms were assessed by tapping the end of each worm with the index finger and applying a bit of pressure. It was also observed for control. The time taken for paralysis, motility activity of any sort, and death time of worms were observed and recorded after ascertaining that the worms did not move neither when shaken vigorously nor when dipped in warm water (50°C).Results were taken in triplicate , Mean ± SD(n=3).

Viscosity

The viscositiy was measured to determine rheological properties of formulations. Brookfield Rheometer viscometer RVDVE at 30°C with a CPE 01 spindle at 30 rpm was used for this purpose. Results were taken in triplicate and the average was taken in to consideration[10].

Globule Size Analysis

Globule size of the formulations was determined using Photon Correlation Spectroscopy (PCS) using a Malvern ZetasizerVer .7.01

Characterization of NEO

Zeta Potential

The zeta potentials of the sample was determined at 25^{o}C after suitable dilution with distilled water using by Photon Correlation Spectroscopy (PCS) using a Malvern ZetasizerVer .7.01.

Percentage Transmittance

The percent transmittance of the nanoemulsion was measured using in a JASCO V-630 spectrophotometer keeping distilled water as blank at 560nm.

Transmission electron microscopy

The morphology of then NEO was examined using by JEOL Model JSM - 6390LV an electronic transmission microscope at 70 kV. After dilution with the original dispersion medium of the nanoemulsion, the samples were negatively stained with 1% (w/v) phosphotungstic acid for observation.

Scanning electron microscopy

The morphology of the nanoemulsion was examined by JEOL Model JSM - 6390LV. The samples were stained with 2% (w/v) phosphotungstic acid for 30 s and placed on copper grids with films for viewing[11].

Comparison of anthelmintic activity

The anthelmintic activity of the formulated product was compared with that of the marketed product micronized Albendazole oral suspension (Bendex suspension,Cipla).The mean time for paralysis and death was found out using the same procedure described before.

RESULTS

Characterisation of Albendazole

Solubility

The solubility of Albendazole in Phosphate buffer pH7.4 containing 1% tween 80 was found to be 0.632mg/ml.

Melting point

The melting point of Albendazole was found to be 213^{0}C.The reported data had a closer value and confirmed the result.

UV-Vis spectroscopy

The UV spectrum of Albendazole[48]in methanol showed a characteristic peak at 242 nm. The reported data had a closer value and confirmed the result.

FT-IR spectroscopy

The FT-IR spectrum of Albendazole[49] showed characteristic peaks at range of 505-3328 cm^{-1}.The prominent peak at 3328.75 cm^{-1} showed N-H stretching. The results observed were in close agreement with the reported data

XRPD

XRPD pattern of Mebendazole showed different peaks 5.14,11.269,19.342,20.58,25.778, 25.969,28.221,28.88 etc.The results observed were in close agreement with reported data.

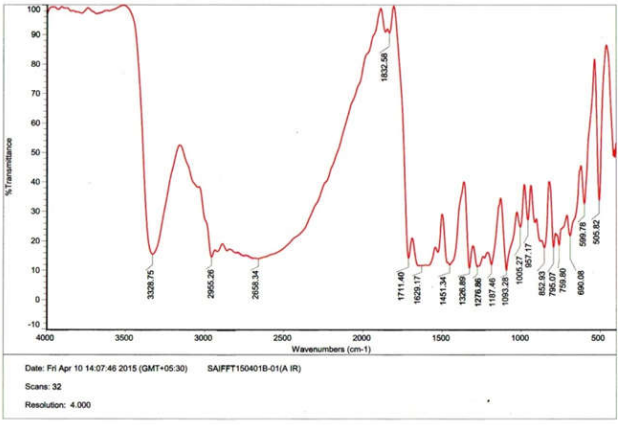

Figure 1 FT-IR of albendazole

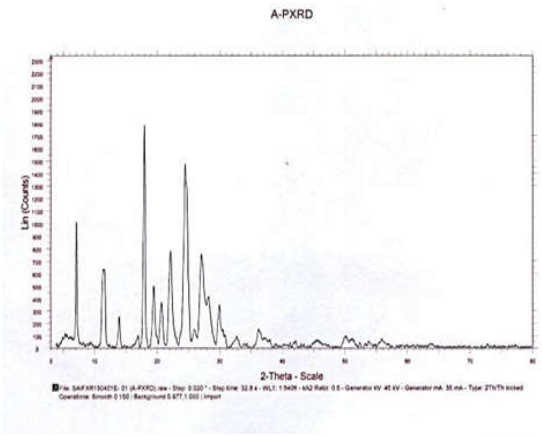

Figure 2 XPRD of albendazole

Characterisation of triacetin

Solubility

Solubility of triacetin was determined qualitatively in various media.

Viscosity

Viscosity of triacetin was determined using Brookfield rheometer viscometer RVDVE at 30°C with a CPE 01 spindle at 30 rpm.

FT-IR spectroscopy

FT-IR spectrumoftriacetin was taken and compared with reference spectrum for confirmation.FT-IR modelThermo Nicolet, Avatar 370was used for obtaining spectra. The spectrum obtained was compared with reported data of triacetin.

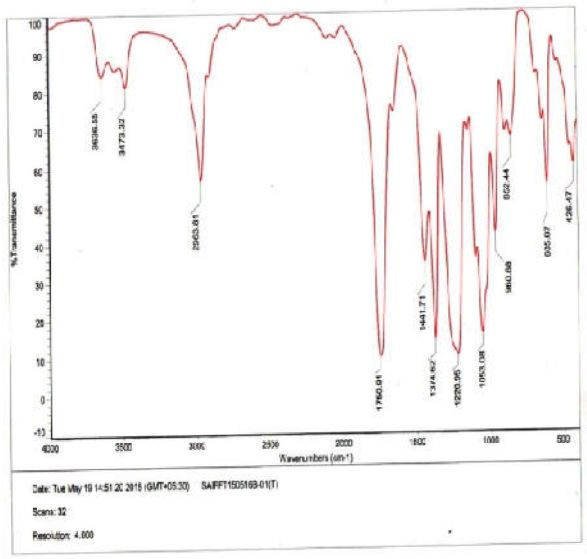

Figure 3 FT-IR of Triacetin

Characterisation of oregano essential oil

Solubility

Oregano essential oil was found to be soluble in ethanol, methanol, acetone, slightly soluble in DMSO, Insoluble in petroleum ether, soluble in phosphate buffer pH 6.8.

Viscosity

Viscosity of Oregano essential oil was found to be 0.8921 Cp.

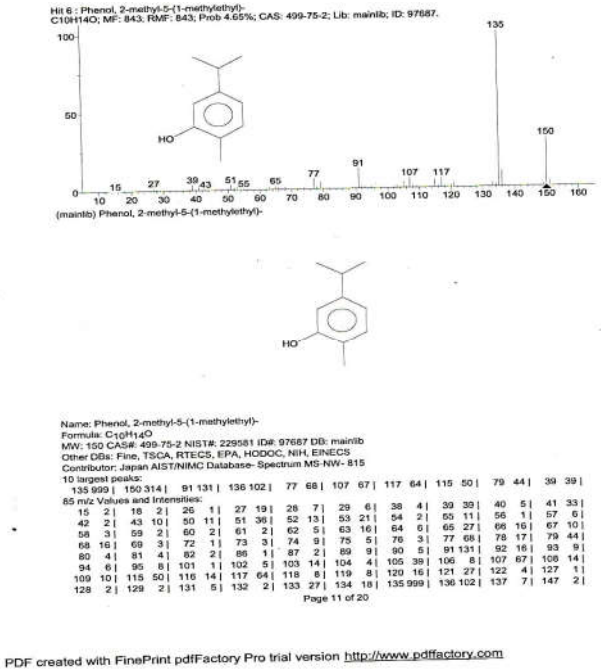

Figure 4 GC-MS of oregano oil

GC-MS

The GC-MS peak for oregano essential oil was obtained. The spectra confirms the presence of phenolic compounds like thymol and cresol. The m/z value representing these compounds was found to be 83.The presence of carvacrol was confirmed in the spectra having m/z value of 55.The results observed were in close agreement with the reported data.

Drug-oil compatability studies

FT-IR spectroscopy

The FTIR spectra of drug and oil phase mixture in the ratio 1:1 was obtained. The FT-IR spectrum of mixture showed characteristic peaks at a range of 426-3625 cm^{-1}.The prominent peak at 3473.75 cm^{-1} showed N-H stretching. The results observed were in close agreement with the reported data.

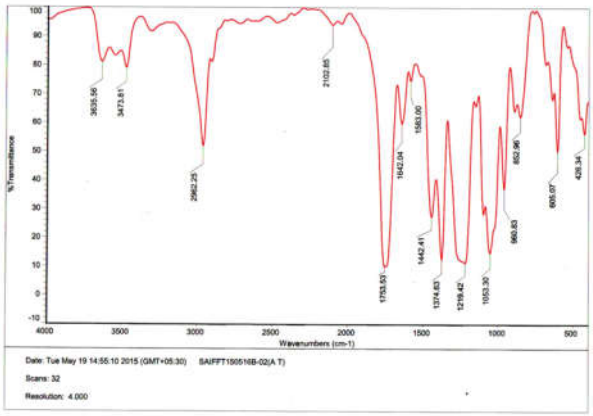

Figure 5 FT-IR of Oil-Drug mixture

GC-MS

The GC-MS peak for oregano essential oil and Mebendazole mixture was obtained. The spectra confirms the presence of phenolic compounds like Thymol and Cresol. The m/z value representing these compounds was found to be 83.The presence of carvacrol was confirmed in the spectra having m/z value of 55.The spectra also confirms the presence of Mebendazole structure fragments like methyl carbamate at 70 m/z and 5-methyl 2-(1-methyl ethyl acetate) at m/z ratio of 63.There was no interaction between the oil and the drug.

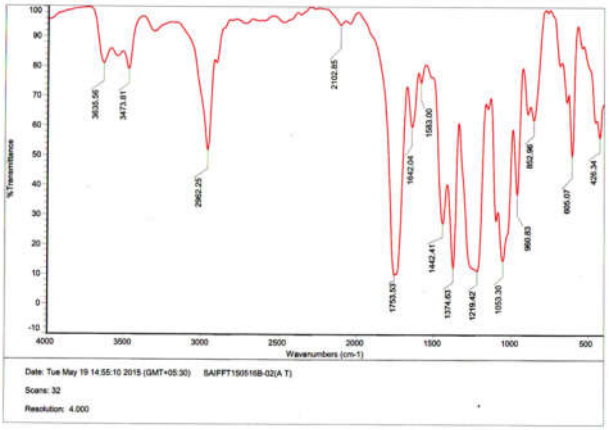

Figure 6 FT-IR of Oil-Drug mixture (1:1)

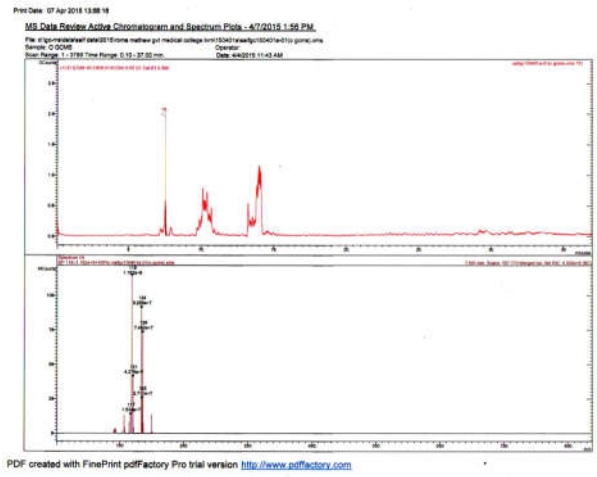

Figure 7 GC-MS of oil-Drug Mixture

Formulation - Design and Development

Pseudo ternary phase diagram

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Study Type	Mixture	Runs	14
Design Type	Simplex Lattice	Blocks	No Blocks
Design Model	Quadratic		

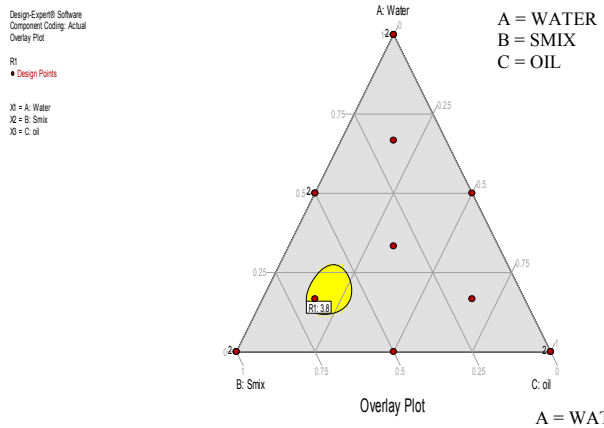

Figure 8 Phase diagram for oil: smix ratio (1:1)

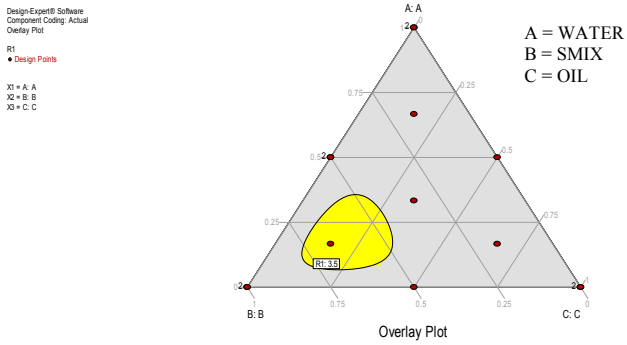

Figure 9 Phase diagram for oil: smix ratio (1:2)

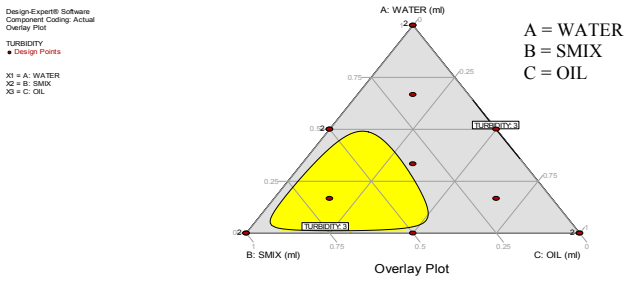

Figure 10 Phase diagram for oil : smix ratio (1:3)

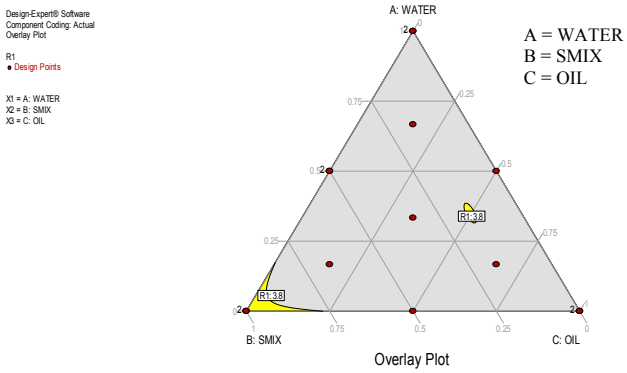

Figure 11 Phase diagram for oil : smix ratio (1:4)

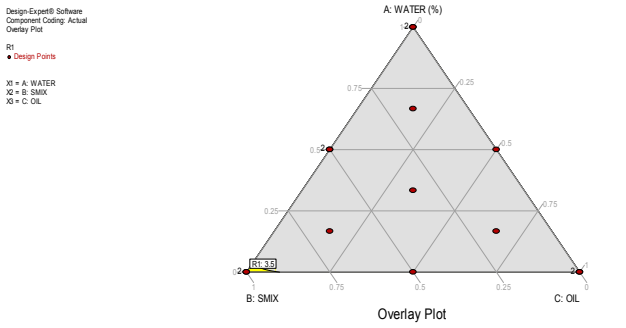

Figure 12 Phase diagram for oil : smix ratio (1:5)

The criteria of choosing respective ratio depends on the area of graphs. The graph showing maximum area is the ratio 1:3 and so was selected.

Optimisation of Nanoemulsion

Anthelmintic activity

Table 2 Anthelmintic activity of nanoemulsion formulation (NEO)

Formulation Code	Mean Time Taken For Paralysis (min)	Mean Time Taken For Death(min)
NEO	1.2±0.01	2.2±0.02

Viscosity

Viscosity of the three formulations NEO was determined.

Table 3 Viscosity of NEO

Formulation Code	Viscosity IN Cp
NEO	18.61 ± 0.45

Globule size

Globule size of the t formulations NEO was determined

Table 4 Observation of globule size and polydispersity index of formulation.

Formulation code	Globule Size nm	Poly dispersity index
NEO	36.24	0.204

Characterisation of optimised nanoemulsion

Zeta potential

Zeta potential of NEO was determined. Increased value of zeta potential prevents the coalescence of globules due to electrostatic repulsion and the value was found to be -53.5 mV.

Percentage Transmittance

The percentage transmittance of NEO was found and was found to be 89.98%.

Transmission electron microscopy

Transmission electron microscopy of NE2 was done and spherical structure of globules were confirmed

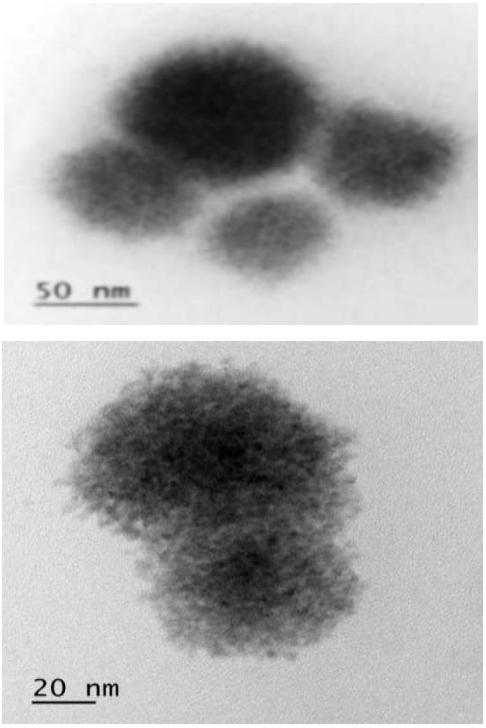

Figure 13 TEM of NEO

Scanning electron microscopy

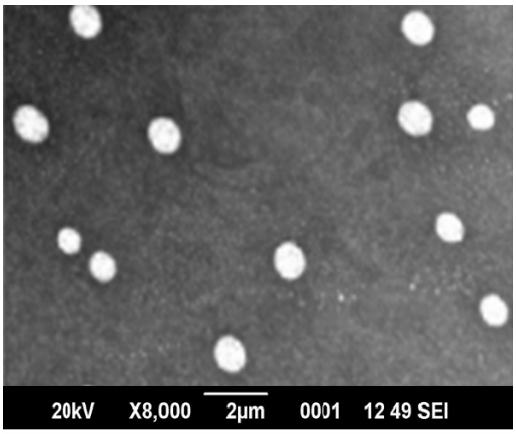

Figure 14 SEM of NEO

Table no.5 Comparison of anthelmintic activity with marketed product

Formulation Code	Mean Time Taken For Paralysis (min)	Mean Time Taken For Death(min)
MARKETED PRODUCT	3.5±0.21	4.2±0.27
NEO	1.2±0.13	2.2±0.20

The determination of anthelmintic activity was performed in triplicate and expressed as Mean ± SD (n=3) The NEO formulation showed greater anthelmintic activity than the marketed product. The mean time taken for paralysis and death was 1.2±0.13 and 2.2±0.20 min respectively.

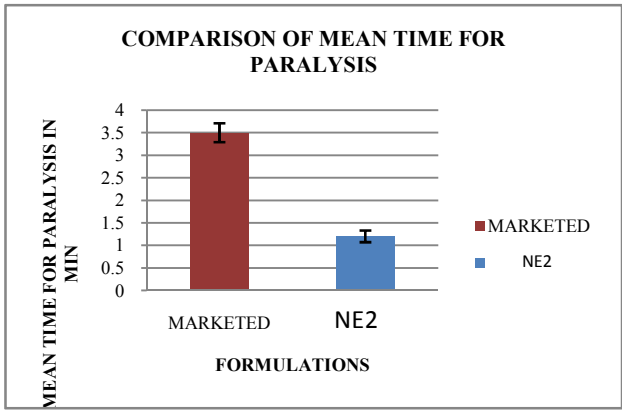

Figure 15 Bar graph representing comparison of mean time for paralysis

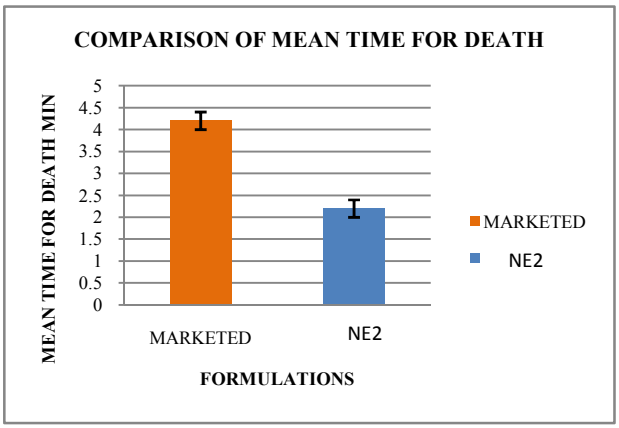

Figure 16 Bar graph representingcomparison of mean time for death

Drug content

Drug content of NEO was determined and reported. The absorbance was measured at 242nm and calculated the drug content using the equation.

Table 6 Comparison of drug content of NEO with marketed product

Formulation	Absorbance At 242 nm	Concentration (mg/ml)	Total amount of drug mg	%Drug Content±SD
NEO	0.2140	0.0895	89.10	89.10±0.014
Marketed Product	0.1725	0.0516	51.60	51.62±0.21

The determination of drug content was performed in triplicate and expressed as Mean ± SD(n=3)

The drug content of NEO was found to be 89.10±0.014 %. The formulated NEO have greater drug content than the marketed product.

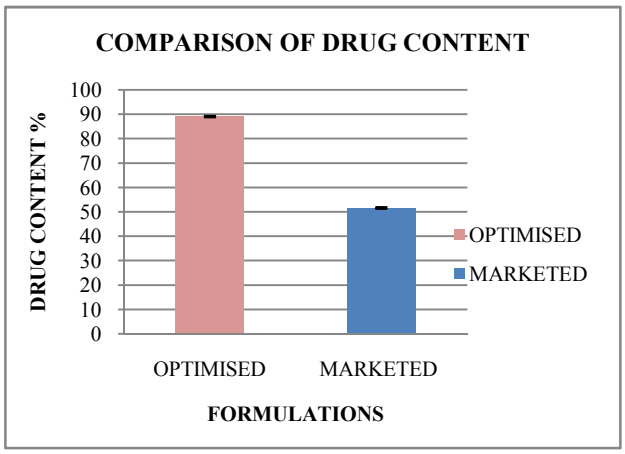

Figure 17 Bar graph representing comparison of drug content

Invitro drug release

The quantitative in vitro release test was performed,using Franz diffusion cell.The samples were analyzed for the drug content using UV-Visible spectrophotometer (Shimadzu, Japan) at 242nm.

Table 7 A comparative study of percentage drug release

Time In Hours	Cumulative Percentage Drug Relea SE±SD	
	NEO	Marketed Product
0	0	0
0.16	4.89±0.04	2.51±0.31
0.3	16.23±1.2	13.01±1.74
0.5	28.59±0.9	24.27±1.12
1	33.03±0.56	29.46±1.45
2	47.69±1.6	38.65±0.9
3	59.91±0.78	48.06±0.36
4	68.63±0.29	57.38±0.89
6	76.07±0.52	69.20±1.10

The determination of cumulative percentage of drug release was performed in triplicate and expressed as Mean ± SD(n=3)

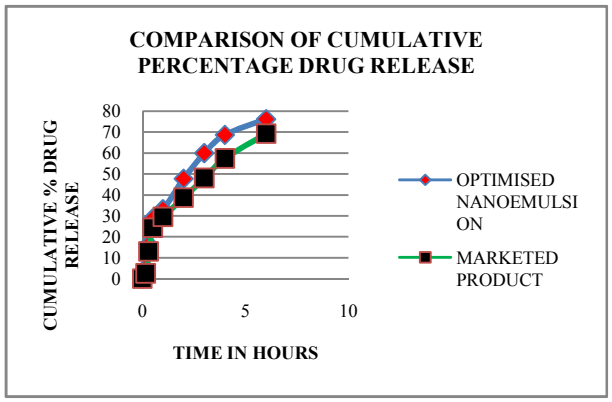

Figure 18 Plot representing comparison of cumulative percentage drug release

Table 8 Comparison of drug content of NEO with marketed product

Time (Days)	Total Drug Content (%) NEO±SD	Total Drug Content of Marketed Product %±SD
0	89.10±0.36	51.43±0.11
30	89.02±0.24	51.01±0.06
60	88.76±0.38	48.26±0.21

The determination of drug content was performed in triplicate and expressed as Mean ± SD(n=3)

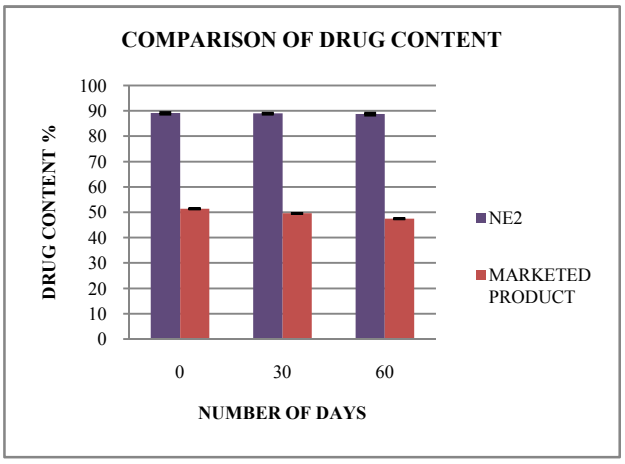

Figure 19 Bar graph representing comparison of drug content of NEO with marketed product

The drug content of nanoemulsion was determined, only a slight deviation in drug content was observed upon storage of NEO over 6o davs.

Table 9 Comparison of cumulative percentage drug release of NEO with marketed product.

Time In	Cumulative % Drug Release ± SD Day 0		Cumulative % Drug Release ±SD Day 30		Cumulative % Drug Release ±SD Day 60	
Hours	NEO	Marketed	NEO	Marketed	NEO	Marketed
0	0	0	0	0	0	0
0.16	4.89±0.04	2.51±0.31	4.53±0.15	1.38±0.31	4.41±0.03	0.09±0.04
0.3	16.23±1.2	13.01±1.74	15.97±0.25	11.56±0.12	15.88±0.24	9.85±0.24
0.5	28.59±0.9	24.27±1.12	27.86±0.03	20.12±0.21	27.21±0.07	17.43±0.12
1	33.03±0.56	29.46±1.45	32.14±0.57	22.31±0.01	31.89±0.22	19.09±0.05
2	47.69±1.6	38.65±0.9	47.52±0.16	32.98±0.23	47.20±0.34	28.31±0.26
3	59.91±0.78	48.06±0.36	58.23±0.05	45.65±0.28	57.99±0.08	40.56±0.23
4	68.63±0.29	57.38±0.89	68.42±0.24	53.99±0.09	68.01±0.056	50.01±0.034
6	76.07±0.52	69.20±1.10	75.32 ±0.54	68.86±0.11	72.56±0.37	62.61±0.16

DISCUSSION

Nanoemulsions are submicron size emulsion. It has higher solubilization capacity than simple micellar solutions and their thermodynamic stability offers advantages over unstable dispersions such as emulsions and suspensions as they can be manufactured with little energy input (heat or mixing) and have a long shelf life. Here a comparative study of formulated nanoemulsion of albendazole using oregano oil was done and that formulated products efficacy was studied with a marketed product. The results proved that that the formulated product had a higher better action than the marketed one. The results of these studies indicated that the albendazole nanoemulsion containing oregano essential oil formulations may be used for enhancing anthelmintic activity, and also suggest that this nanoemulsion is a promising a novel formulation that can

enhance the solubility of poorly soluble drug albendazole and thereby enhance its oral bioavailability.

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