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RESEARCH ARTICLE

FROSTBITE WOUND HEALING ACTIVITY OF IBUPROFEN NANOEMULSION BASED GEL WITH THE FRACTIONAL DOSE FOR EXTRACTION OF *MIMOSA PUDICA* LEAVES.

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Abstract

The main aim of this study is to determine formulation, Development and Optimization of novel nanoemulsion formulation and treatment of frostbite injury with the fractional dose of *Mimosa pudica* leaves. Materials Ibuprofen as active ingredient was collected from the Merck Specialities private Ltd. Oleic acid and glycerol, and PEG 400, was collected from the Merck Specialities Private Ltd, Ethanol was collected from China, (Changshu Yangyuan Chemicals), Petroleum ether parches from the Merck Specialities private Ltd, Extract Raw material *Mimosa pudica* was collected from the Azara, Guwahati local area and air dried and powered the leaves. Nanoemulsion prepared by high pressure homogenization method. Characterization of Nanoemulsion was done to determine the thermodynamic Stability, Dynamic Light Scattering Spectrophotometer, Zeta Potential, Transmission Electron Microscopy (TEM), Drug Content, Viscosity Measurement and in-vitro drug released. It shows as good characteristic of nanoemulsion and can be predicted particle range from 200 to 252 nm. Zeta potential conductivity 0.0910 mV to 0.00363 mS/ Cm found for nanoemulsion formulation. Apart from these SEM and TEM was carried out which shows very small particle and well distribution. Irritation and antinflammatory done by direct applying the formulation topically and observed for 15 to 20 days.

Introduction:-

Nanoemulsion is a heterogeneous system and it consists of two immiscible phase, one phase is oil phase other is aqueous phase, while the droplet is submicron size range of 20-200nm. It is thermodynamically stable, optically clear, transparent formulation of nanoemulsion[1]. Now-a-day's nanoemulsions are frequently used for various purpose like delivery of vaccine, DNA encoded drug antibiotics, cosmetic and topical preparations and can be administrated via various routes like oral, pulmonary, ocular transdermal, Intranasal, biodegradable polymeric nanoparticles used as drug delivery systems[2,3].Nanoemulsion, which is categorized as multiphase colloidal dispersion is generally characterized by its stability and clarity. The dispersed phase typically comprises small particles or droplets and has very low oil/water interfacial tension. Nanoemulsion is formed readily and sometimes spontaneously, generally without high-energy input. In many cases a cosurfactant or co-solvent is used in addition to the surfactant, the oil phase and the water phase. Oil-in-water emulsions are most useful as water washable drug bases and for general cosmetic purposes, while water-in-oil emulsions are employed more widely for the treatment

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of dry skin and emollient applications. Gels for dermatological use have several favourable properties such as being thixotropic, greaseless, easily spreadable, easily removable, emollient, non-staining, compatible with several excipients and water-soluble or miscible. Emulgels are emulsions, either of the oil-in-water or water-in-oil type, which are gelled by mixing with a gelling agent. They have a high patient acceptability since they possess the previously mentioned advantages of both emulsions and gels. Therefore, they have been recently used as vehicles.

Types of Nanoemulsion:-

Nanoemulsion are classified into mainly three types on the basis of their composition

1. Oil in water Nanoemulsions:

In oil in water nanoemulsion oil dispersed in water. Hence dispersion medium is water and this kind of nanoemulsion can be found in application which has small quantity of fatty material like moisturizing, creams. Example: milk which is also like these types of emulsion.

2. Water in oil Nanoemulsions:

In case of the Water in Oil Nanoemulsion water droplets are found to be dispersed in continuous oil phase.

3. Bi-continuous Nanoemulsions:

In case of bi- continuous nanoemulsion oil and water are interdispersed within the system.

Skin:-

The human skin is the outer covering of the body. In humans, it is the largest organ of the integumentary system. The skin has up to seven layers of ectodermal tissue and guards the underlying muscles, bones, ligaments and internal organs. Human skin is similar to that of most other mammals. Though nearly all human skin is covered with hair follicles, it can appear hairless. There are two general types of skin, hairy and glabrous skin. The adjective cutaneous literally means "of the skin" (from Latin *cutis*, skin) In humans, skin pigmentation varies among populations, and skin type can range from dry to oily. Such skin variety provides a rich and diverse habitat for bacteria that number roughly 1000 species from 19 phyla, present on the human skin.[4,5] Skin is composed of three primary layers: the epidermis, the dermis and the hypodermis. Different layer of skin is shown in Fig 1.

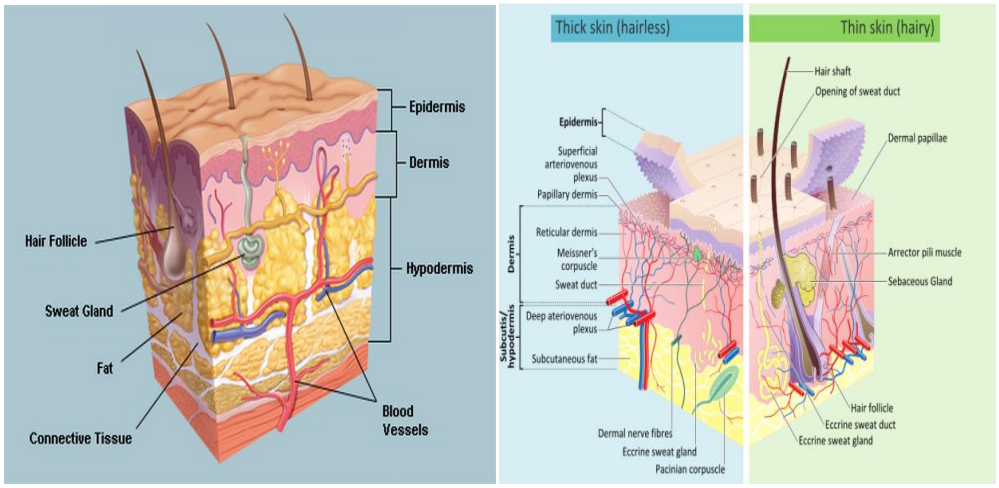


Fig 1:- A) Skin's layer; B) Skin layer without hair and with hair

Frostbite:-

Frostbite is an injury that is caused by exposure of parts of the body to the cold. The cold causes freezing of your skin and underlying tissues. Your fingers, toes and feet are most commonly affected. There are different degrees of frostbite. In superficial frostbite, the skin can recover fully with prompt treatment. However, if frostbite is deep, tissue damage can be permanent and tissue loss can occur. For example, the end of a finger or toe can gradually separate off. The most important way of preventing frostbite is to get out of the cold. If you are exposed to the cold, make sure that you have adequate protective clothing. Frostbite is an injury that is caused by exposure of parts of your body to temperatures below freezing point. The cold causes freezing of your skin and underlying tissues. The fingers, toes and feet are most commonly affected but other extremities including the nose, ears, and the cheeks can also develop frostbite[6].

Degrees of frostbite:-

Rather like burns, frostbite injuries are classified by the degree of injury. The degree of frostbite basically refers to how deep the frostbite injury goes. Your skin has two layers - the outer layer (epidermis) and the dermis. The dermis sits just under the epidermis. Beneath the dermis is a layer of fat, and then the deeper structures such as muscles and tendons.

- First-degree frostbite just affects the epidermis.
- Second-degree frostbite may affect the epidermis and part of the dermis.
- Third-degree frostbite affects the epidermis, the dermis and the fatty tissue beneath the dermis.
- Fourth-degree frostbite affects the full thickness of the skin, the tissues that lie underneath the skin, and also deeper structures such as muscles, tendons and bone. Different type of frostbite injury indicated in Fig.2.

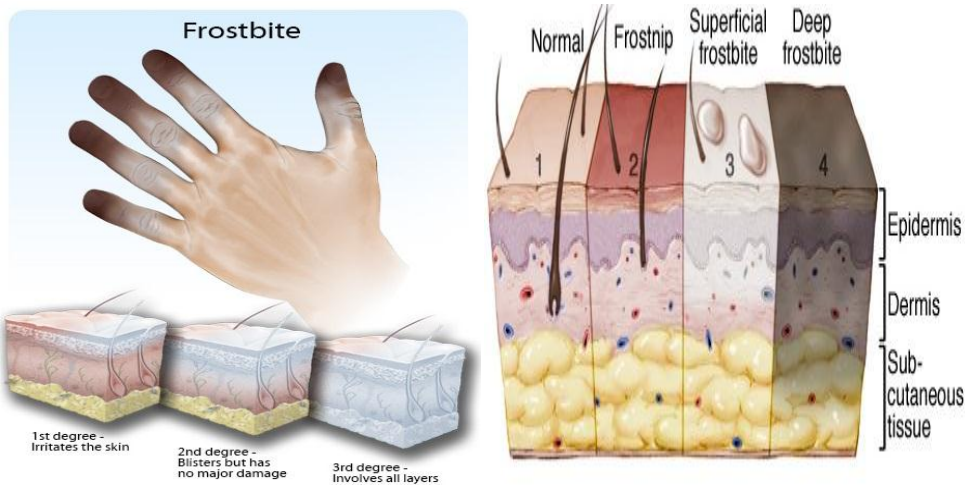


Fig.2:- Degree of frostbite in epidermis dermis and subcutaneous tissues of skin

Extraction of *Mimosa pudica* leaves:-

Extraction in chemistry is a separation process consisting in the separation of a substance from a matrix. It includes Liquid-liquid extraction, and Solid phase extraction. A Soxhlet extractor is a piece of laboratory apparatus[7] invented in 1879 by Franz von Soxhlet[8].It was originally designed for the extraction of a lipid from a solid material. Typically, a Soxhlet extraction is used when the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. It allows for unmonitored and unmanaged operation while efficiently recycling a small amount of solvent to dissolve a larger amount of material. *Mimosa pudica* leaves. is a creeping annual or perennial herb. It has been identified as lajjalu in Ayurveda and has been found to have antiasthmatic, aphrodisiac, analgesic, and antidepressant properties. *M. pudica* is known to possess sedative, emetic, and tonic properties, and has been used traditionally in the treatment of various ailments including alopecia, diarrhea, dysentery, insomnia, tumor, and various urogenital infections. Phytochemical studies on *M. pudica* have revealed the presence of alkaloids, non-protein amino acid (mimosine), flavonoids C-glycosides, sterols, terpenoids, tannins, and fatty acids[9]. *Mimosa pudica* leaves was shown in Fig 3.

Material And Method:-

Materials:-

Materials Ibuprofen as active ingredient was collected from the Merck Specialities private Ltd. Oleic acid and glycerol, and PEG 400, was collected from the Merck Specialities Private Ltd, Ethanol was collected from China, (Changshu Yangyuan Chemicals), Petroleum ether parches from the Merck Specialities private Ltd, Extract Raw material *Mimosa pudica* was collected from the Azara, Guwahati local area and air dried and powered the leaves.

Fig 3: Leaves of Mimosa pudica leaves

Methods:-
Preformulation Study:-
Organoleptic Properties:-
Organoleptic properties was determined by the visually, to determined the different colour, odour and taste of the sample.

Solubility Study by Different Solvents:-
The most important criteria for screening of excipients is the solubility of the poorly soluble drug in oil, surfactants, and co-surfactants. Solubility of the Drug Ibuprofen was carried out with different solvent by taking small amount of the sample to the test tube and shake well to observe if any crystal or precipitation form.

Melting points:-
Small amount of sample inserted to the capillary tube, attaching to the stem to the Thermometer to the centre in a heating bath, heating the bath slowly and observed the temperature at which the sample started melting is pointed, pure sample usually have sharp melting point. The melting point of Ibuprofen was found to 76°C.

Differential Scanning Calorimetric:-
Drug and excipients weighted between 5 -10 mg and kept in the desiccators for 24 hrs. The sample was than kept in the sample holder and analysed was perform.

FTIR (Fourier transform infrared spectroscopy):-
Drug excipients and their physical mixture of smal amount were taken in the mortor and mixed well to form uniform mixture and placed to the deccicator if required and perform FTIR at scanning with 400 cm^{-1} to 4000cm^{-1}.

Thin Layer Chromatography [IP]:-
Silica gel plate:-
Silica gel plate was prepared by Pouring method where the powder silica gel of approximately 30 gm dissolved in 60ml of water and forms the slurry which is poured to the glass plate and kept for drying at 120°c for 30 min for activation of the plate to remove the water.

A] Mobile Phase:-
A mixture of 75 volume of n hexane 25 volume of Ethyl Acetate and volume of glacial acetic acid.

B] Test Solution:-
Dissolved 0.5 mg of substance with 100 ml of Dichloromethane. Apply to the plate 5µl of each solution was placed and dry at 120°c for 30 minute and lightly spray with 1% w/v of potassium permanganate in 1M Sulphuric acid and observed under UV light at 365nm.

Extraction of *Mimosa pudica* leaves:[10, 11, 12]:-

Mimosa pudica (Lajjalu) has been extensively used in ayurveda, unani & homoeopathic medicine and has become cynosure of modern medicine. It is known in sensitive or humble plant & popular name is lajjavanti & chuimui. Punjabi name of this plant is lajwanti *Mimosa pudica* posses a wide area of therapeutic activity likes vulnerary, diuretic, antispasmodic, emetic, constipating and febrifuge. They used in haemorrhage, dysentery, inflammation, burning sensation & wounds. It is also used in jaundice, asthma, conjunctivitis, cut wounds, and glandular swelling[13, 14,15,16,17]. The present study has been undertaken to examine the wound healing activity of the leaves of *Mimosa pudica* in excision and burn wounds models Preparation of leaves extract- The leaves of *Mimosa pudica* Linn were collected from local market, area. Powdered leaves were charged into soxhlet apparatus and successive hot continuous extraction was carried out using solvents such as Petroleum ether (600-800 0 C), and ethanol. The drug was extracted with each solvent. Each time before extraction with the next solvent, the powdered material was air dried. Each extract was concentrated by distilling the excess solvent to obtain the crude extract.The Petroleum Ether was used to remove the fat and tannins of the *Mimosa pudica* leaves than mark was kept for drying over night and carried out with solvent ethanol. Concentrated the ethanol and collected the extract. Process of *Mimosa pudica* leaves was have been shown in Fig.4.

Phytochemicals screening of *mimosa pudica* leaves: (Dey and Raman 1957)[18]

Dilution:-

Dilution of *Mimosa pudica* leaves extract was performed in 1ml of extract with 100 ml of ethanol

Test for Tannins:-

Preparation of 0.1% ferric chloride:-

To 99.9 ml of distilled water 0.1ml of ferric chloride reagent was added.

Table 1:- Phytochemical screening of *Mimosa pudica*

Sl. No.	Test	Observation	Inference
1.	**Ferric chloride Test** 1 ml of the sample taken and a few drops of 0.1% ferric chloride was added	Observed for brownish green or blue, black colouration.	Tannins present
2.	**Test For Saponins** To 1 ml of extract 5 ml of distilled water was added and shaken vigorously. Observed for soaking appearance indicates the presence of saponins.	Observed for soaking appearance indicates the presence of saponins	Saponins Present
3	**Test For Flavonoids** To 1 ml of extract 5 ml of dilute ammonia solution was added, followed by addition of concentrated sulphuric acid along the sides of the tube	Appearance of yellow colouration	Flavanoids Present
4	**Test for Alkaloids** 1 ml of sample was taken to that few drops of Dragandoff reagent was added and	Observed for orange red colour.	Alkaloids present
5	**Test for Anthroquinones** 1 ml of sample was taken to that aqueous ammonia (shaking) was added[18]	observed for change in colour of aqueous layer (Pink, Red or Violet)	Anthroquinones present

Determination of flavanoid in *Mimosa pudica* Leaves:

Aluminium chloride colorimetric method was used for flavonoids determination. 1 ml of sample was mixed with 3 ml of Ethanol, 0.2 ml of 10% aluminium chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a UV/Visible spectrophotometer. The calibration curve was prepared by preparing Quercetin solutions at concentrations 12.5 to 100 µg/ml in Ethanol[19].

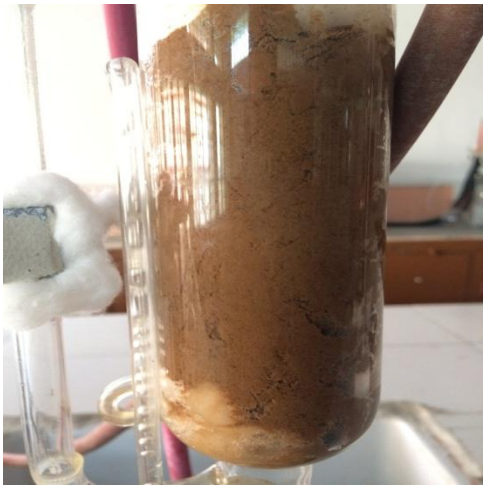


Fig 4:- Process for extraction of *Mimosa pudica* leaves by soxhlet apparatus

Preparation of Nanoemulsion:-

The formulations were prepared by mixing appropriate amount of surfactant and co-surfactant and then oily part added, mix the formulation until completely dispersion occurs at room temperature. Then appropriate amount of drug was added and the *Mimosa pudica* extraction was dissolved with ethanol, transfer to the final mixture and mixed by Homogenizer for 5 hrs continuously.[21]After swellings of whole night the carbopol gel withdrawn and ibuprofen with extraction of *Mimosa pudica* was slowly added to the nanoemulsion with magnetic stirred and nanoemulsion gel was placed in well closed container for further studies.

Table 2:- Different concentration of ibuprofen and other polymer used in formulation

Sl. No.	Formulation code	Quantity					
		Ibuprofen	**Oleic acid**	**Glycerol**	**PEG 400**	**Extract M.P**	**D.W**
1	F1	2.5%	50 %	25 %	10 %	5%	QS
2	F2	2.5 %	50%	25%	10%	3.75%	QS
3	F3	2.5 %	50%	15%	10%	5%	QS

**QS: Quantity Sufficient*

Characterization of Nanoemulsion:-

Physical appearance:-

Nanoemulsion with the fractional dose of Mimosa pudica is green in colour due to the extraction it does not show any turbidity.

Thermodynamic Stability:-

Thermodynamic Stability Studies: Nanoemulsion was subjected to various storage conditions of temperature and humidity to assess their stability as per ICH guidelines Q1A (R2) Physical and chemical stability of nanoemulsion were evaluated for six months by storing them at 30 ± 20C / 65 ± 5% RH and 40 ± 20C / 75 ± 5% RH To overcome the problem of metastable formulation, thermodynamic stability tests were performed. Formulations centrifuges at 3500 rpm for 30 minutes. Those formulations that does not show any phase separations take for the heating and cooling cycle. Six cycles between refrigerator temperatures of 4ºC and 45ºC for 48 hours. The formulations stables at these temperatures are subjected to the freeze-thaw cycle test. Three freeze-thaw cycles do for the formulations between –21ºC and +25ºC. Those formulations that survive thermodynamic stability tests are select for the further studies[20].

Particle size determination:-

Droplet size distribution of the nanoemulsion was determined by photon correlation spectroscopy that analyzes fluctuations in light scattering due to Brownian motion of the particles, using a Zetasizer 1000 HS (Malvern

Instruments, UK). The formulation (0.1 ml) was dispersed in 50 ml of water, mixed thoroughly and light scattering was carried out at an angle of 90 at 25˚C.

Drug Content:-
Certain amounts of nanoemulsion was taken dissolved using 100ml of ethanol and sonicated for the period of 15 min filtered it by whatman filter paper. Further dilutions were made by using ethanol prepared concentration within Beer's range. The absorbance was measured by UV-Visible spectrophotometer and drug content was determined. Apart from this Western Blot method is used to measured amount of drug present.

Viscosity Determination:-
Viscosity of nanoemulsion should be measured by using the rotary viscometer at different rate and temperature. Nanoemulsions have very low viscosity[21]. Brookfield viscosity usually refers to a viscosity measurement performed with a Brookfield Viscometer. There are several models of viscometer available from Brookfield but the majority operate in the same manner: the viscometer motor rotates the spindle at a defined speed (measured in rpm) or shear rate and the viscometer measures the resistance to rotation and reports a viscosity value. Various spindle designs can be employed, depending on the nature of the sample and the requirements.

pH Determination:-
pH of the nanoemulsion was determined by litmus paper and pH meter which is maintained at 5 to 6 which is the skin pH due to that there is no skin irritation.

Zeta Potential:-
In most cases, colloidal particles possess a positive or negative electrostatic charge. As electrical fields are applied to the particle dispersion, the particles migrate in oppositely charged directions. As particles are irradiated in migration, scattering light causes Doppler shift depending on electrophoresis mobility. NanoPlus software calculates the amount of Doppler shift followed by electrophoretic mobility and zeta potential by combining a heterodyne system and photon correlation method to perform Fourier transforms (FFT) Slipping level Major part of medium of obtained correlation function.

Transmission Electron Microscopy:-
Morphology and structure of the nanoemulsion were studied using transmission electron microscopy TOPCON 002B operating at 200 KV (Topcon, USA) and capable of point to point resolution. Combination of bright field imaging at increasing magnification and of diffraction modes was used to reveal the form and size of nanoemulsion droplets. In order to perform the TEM observations, a drop of the nanoemulsion was directly deposited on the holey film grid and observed after.

Scanning electron microscopy:-
Scanning Electron Microscopy uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. In most SEM microscopy applications, data is collected over a selected area of the surface of the sample and a two-dimensional image is generated that displays spatial variations in properties including chemical characterization, texture and orientation of materials. The SEM is also capable of performing analyses of selected point locations on the sample. This approach is especially useful in qualitatively or semi-quantitatively determining chemical compositions, crystalline structure and crystal orientations.

***In-vitro* drug Release:-**
The *in-vitro* drug release studies were carried out using a Franz diffusion cell. The formulation was applied the surface of egg membrane which was placed between donor and receptor compartment of the Franz diffusion cell. Phosphate buffer pH 5.5 was used as a dissolution media. The temperature of the cell was maintained at 37^0 C by circulating water jacket. This whole assemble was kept on a magnetic stirrer and the solution was stirred continuously using a magnetic bead. Sample (1 ml) was withdrawn at suitable time intervals and dilute up to 10ml with same solvent and replaced with equal amounts of fresh dissolution media. Samples were analyzed spectrophotometrically at 263 nm and the cumulative % drug release was calculated[22].

Development of frostbite injury and animal models:-
Preparation of Skin:-

Before day 0, Rats were individually anesthetized By using chloroform The skin surface from the base of the neck to the top of the rear haunches was shaved with an electric clipper, after which a depilatory cream (Nair, Church & Dwight, Princeton, NJ) was applied for 2 minutes to remove any remaining hair. After depilation, the skin was cleansed with a 70% isopropyl alcohol swab.

Continuously freezing method:-

Four Rats were studied using the CF method. Twenty four hours after skin preparation, ceramic (ferrite) magnets (diameter 0.5 inches, thickness 0.219 inches, weight 3.5 g) were placed in crushed dry ice (-78.5°C) and allowed to cool for 15 minutes. The centre of the tattooed circle was marked to determine precisely the placement of magnets. Using fingers, the back skin of the rats was lifted into a skin fold, and then, 2 frozen magnets were placed so that they adhered from opposite sides of the intervening skin fold with the mark at the fold's apex centre location. A silicone barrier was then slid underneath the magnets as a thermal shield to limit the rat's body temperature decline. Sets of 2 cooled magnets were left in place for 1 minute, and then removed to allow new magnets to be immediately placed in the same location against the frozen tissue. The magnet exchange occurred in less than 5 seconds, which did not allow any thaw to occur. The magnet exchange was repeated for a total of 5 applications, resulting in a freeze time slightly longer than 5 minutes. Injury was intentionally inflicted on a lifted skin fold rather than by applying topical pressure and freezing the tissue in its normal position directly underneath the magnet to simplify the method and avoid between-rat variability, confine the injury to the skin, create a precise injury, limit systemic hypothermia, and maximize survival. It was not the intention to create a wound below the dermis. Core temperature was monitored using an infrared thermometer (ThermoWorks, Alpine, UT) applied to the abdomen of therat. After 5 magnet placements and removals, the skin was allowed to completely thaw; Rats were given subcutaneous injections of buprenorphine (0.05 mg/kg) for analgesia after the thaw. No dressings were applied to the wounds[22, 23].

Results and Discussion:-

Preformulation Studies:-

Organoleptic Properties:-

The result of the visual observation of the pure drug ibuprofen was found to be white crystalline and bitter in taste.

Solubility Study by Different Solvents:-

The solubility of Ibuprofen was found as insoluble with water but soluble with some other organic solvents like ethanol, acetone, at the same time ibuprofen was partially in soluble with pH solution of 6.8 and 7.4 respectively.

Table 3:- Solubility studies for ibuprofen and chitosan

Chemical	SOLVENTS	OBSERVATION
Ibuprofen	Water	Insoluble (Slightly soluble)
	Ethanol	Soluble
	Acetone	Soluble
	Buffer pH 7.4	Partially soluble
	Buffer pH 6.8	Partially soluble
Chitosan	Glacial acetic acid	Soluble

Melting Points of Ibuprofen:-

The melting points of Ibuprofen were found to 76°C

Differential Scanning Calorimetric.

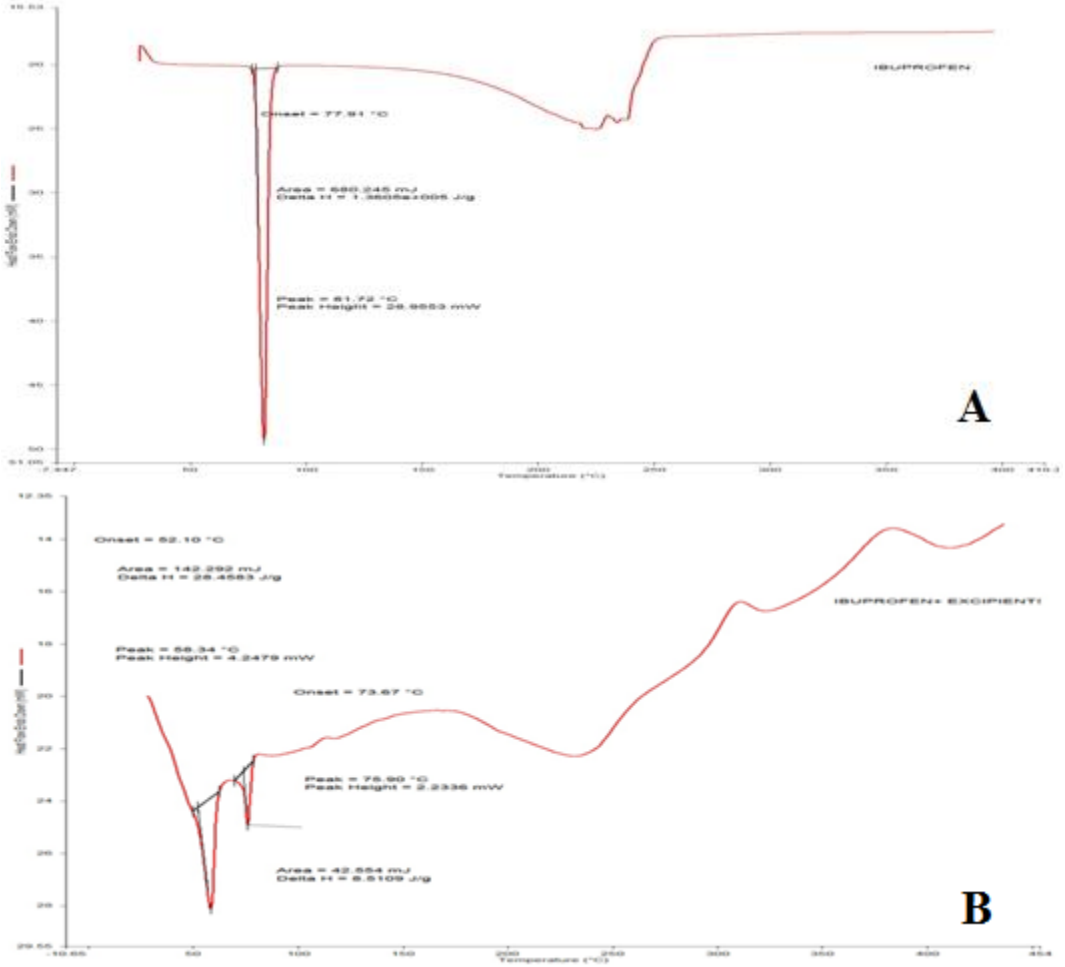


Fig 5:- DSC curve for A) Ibuprofen and B) Ibuprofen+polymer

DSC study of pure Ibuprofen and along with the drug and excipients was performed already mentioned methods and presented in figure no.5. Sharp endothermic peak at 82.72^{0}C which is near about standard value, While the DSC thermogram of the drug along with excipients showed three distinguished peaks at 58.34, 75.9, 241.7 ^{0}C of PEG 4000, Ibuprofen and Carbopol 934 Respectively. From this curve it can be observed that the drug and all the excipients shown their respective different peaks which state the confirmation of the compatibility of the drug along with the excipients used in formulation.

FTIR (Fourier transform infrared spectroscopy:-

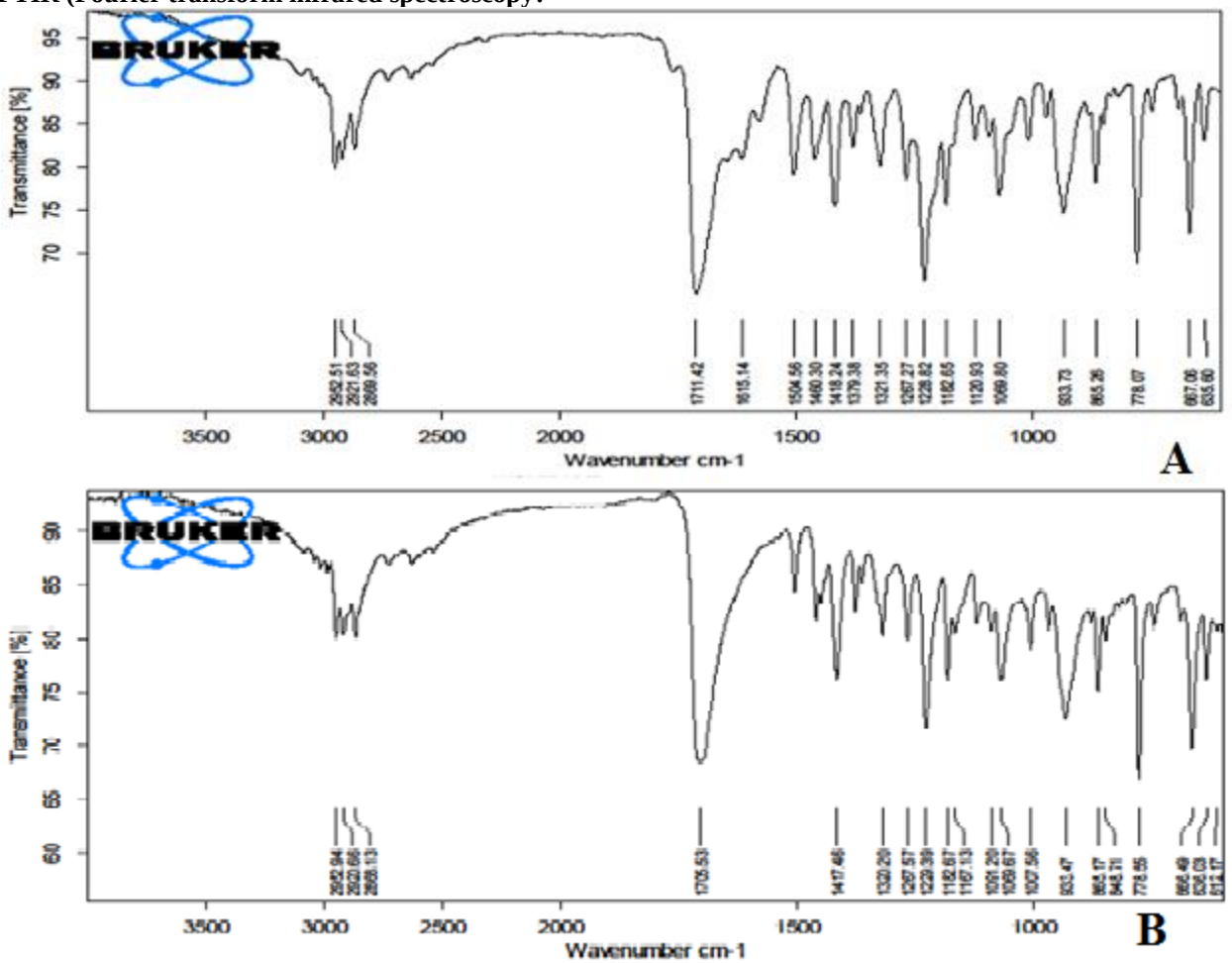


Fig 6:- FTIR Spectra for Ibuprofen and other polymer

Table 4:- IR spectra analysis of Drug

Fuctional group	IR Absorption cm^{-1}
CH_3	2850-2960
Amino acid, NH	3931.64
Primary Amin, NH	3733.45
Aldehyde,	2650-2880
Aromatic ring –c-c-	1505
Alkane	2991.43
CH	2725.35

Extraction of *Mimosa pudica* leaves:-

Mimosa pudica was extracted by using Petroleum ether and ethanol. It is defined as the alcoholic extract where the petroleum ether was used to remove the tannins and fat in the leaves. Ethanol gives the phytochemical screening of flavanoids and other constituents.

Phytochemical Screening of *Mimosa pudica* leaves:-

After extraction of *Mimisa pudica* leaves Phytochemical analysis was carried out for different constituents. As standard methods of Dey and Raman 1957 applied and found that extraction contains of Tannins but less amounts, Saponins (less amount) Flavanoids, alkaloids and Anthroquinones.

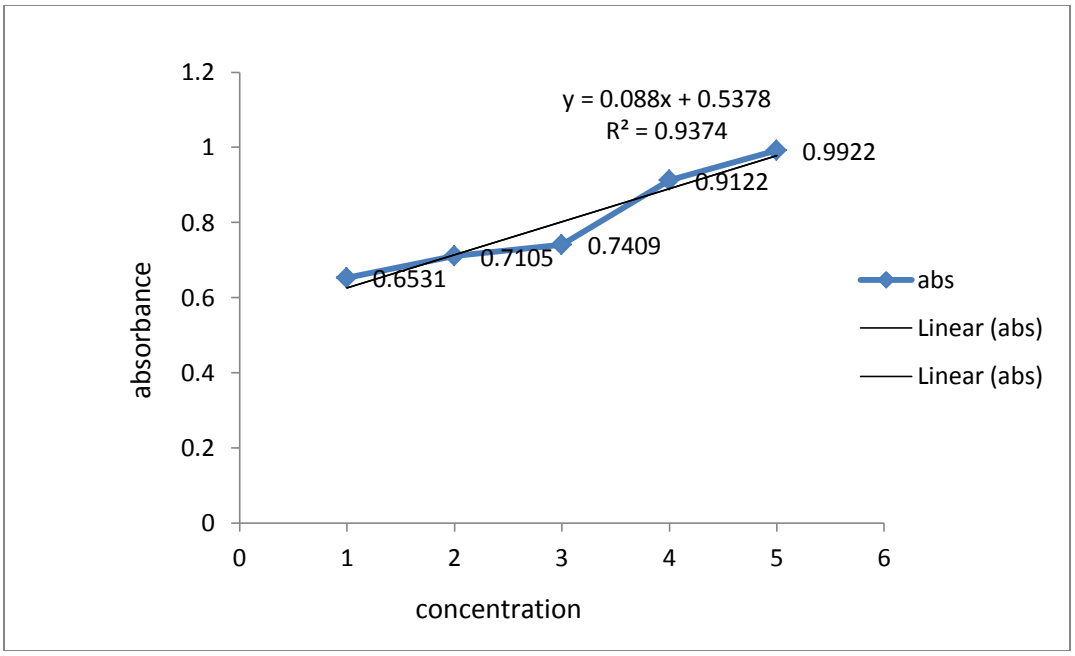


Fig 7:- Curve for the extraction of *Mimosa pudica*

Determination of total flavanoids presents in extraction of *Mimosa pudica* leaves:-

Aluminium chloride colorimetric method was used for flavonoids determination. First of all the quercetin standard curve was prepared and compared with the extracted U.V absorption.

Concentration of flavanoids present in *Mimosa pudica* leaves Standard Curve was prepared from the absorption values put on graph at different concentration from 16, 32, 48, 64, 80 and 96 µg/ml. The total flavanoids concentration in *Mimosa pudica* leaves is being calculating by Quercetin standard curve (QE) Y=0.088x+0.537, r^2 = 0.937 and after combining the absorbance value of extraction of leaves it founds that y=0.089x, r^2= 0.989. The concentration of flavanoids is found to be 151.2 µg/ml.

Characterization of nanoemulsion formulation:-

Physical appearance:-

Nnaoemulsion with the fractional dose of *Mimosa pudica* is green in colour due to the extraction present in formulation; it does not show any turbidity.

Table 5:- Thermodynamic stability study:

Sl. No.	Formulation	Heating cooling cycle	Centrifugation cycle	Freeze Thaw cycle	Inference
1.	F1	Yes	yes	yes	Stable
2.	F2	Yes	yes	yes	Stable
3.	F3	Yes	yes	yes	Stable

From the above study, it was concluded that formulation 1, 2 and 3 found to be thermodynamically stable.

Table 6:- Particle size determination (Zetasizer):

F.Code	Particle size	PdI	Homogenization Time (hour)	Homogenization Speed (rpm)
F1	203.9	0.258	5	11000
F2	235.2	0.391	3	13000
F3	229.9	0.309	5	12000

It was concluded that peak was shown at the particle size range of 203.9 to 229.9 nm and the graph depict that it has a homogeneous distribution of particles. Thus the result showed that the particle size of formed nanoemulsion was in the required range, therefore nanoemulsion formulated successfully.

Drug Contain determination:-

Drug contain found as in the range of 88 % to 98%.

Table 7:- Drug content determination of nanoemulsions

Drug	F1	F2	F3
Ibuprofen	88 %	90 %	98%

Ibuprofen loaded nanoemulsion drug contain was determined by U.V analysis which found F3 as highest % of drug contained. It shows in table no.7

Viscosity Determination:-

Table 8:- Determination of viscosity

Sl	Speed (rpm)	Viscosity (cp) F1	Viscosity(cp) F2	Viscosity(cp) F3
1	0	0	0	0
2	5	1300	1430	1250
3	10	300	420	390
4	20	210	315	350
5	50	120	150	250
6	100	30	65	75

Viscosity was determined by Brookfield viscometer of 64 spindle in different rotation where the share-stress applied viscosity decreases which shows the thixotropic behaviour of the gel, which means after applying the stress gel convert to solution. It states that gel are having good viscosity. Result shown in table no. 8

pH Determination:-

The pH of gel in between 5 to 6 which lies in between normal pH range of skin which does not produce any skin irritation.

Table 9: pH of different formulations

Sl	Formulation	pH
1	F 1	5
2	F2	6
3	F3	5

Zeta potential:-

Zeta potential 0.0910 mV to 1.02 mV for formulation 1,2 and 3. Where conductivity 0.00363 mV to 0.00371 mS/Cm for different formulation which is being shown in table no.10

Table 10:- Zeta potential of the formulations

F. Code	Zeta potential (mV)	Conductivity (mS/Cm)
F1	0.910	0.00363
F2	0.976	0.00369
F3	1.02	0.00371

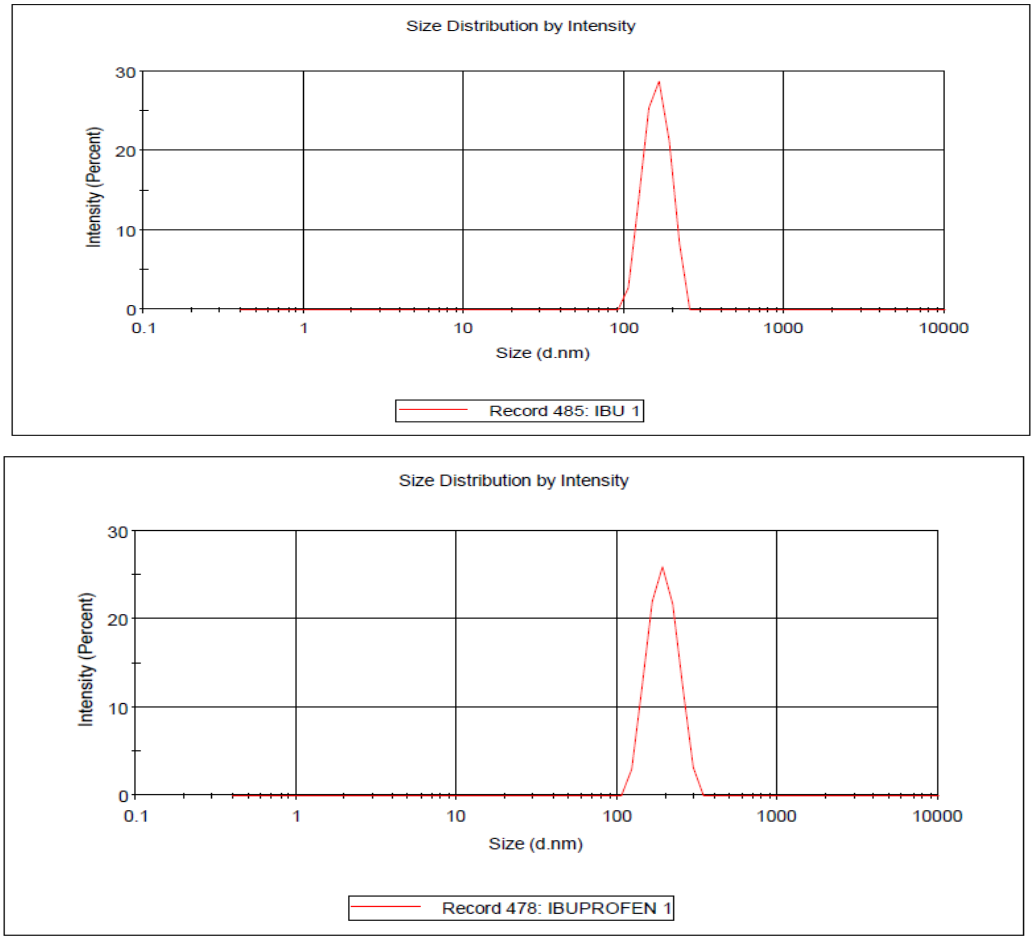
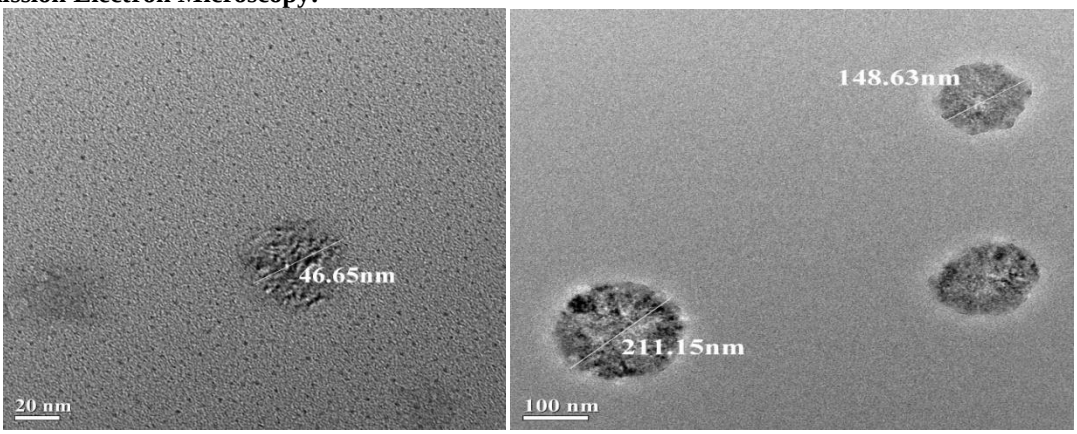


Fig:- 8 Curve for Zeta potential

Transmission Electron Microscopy:-



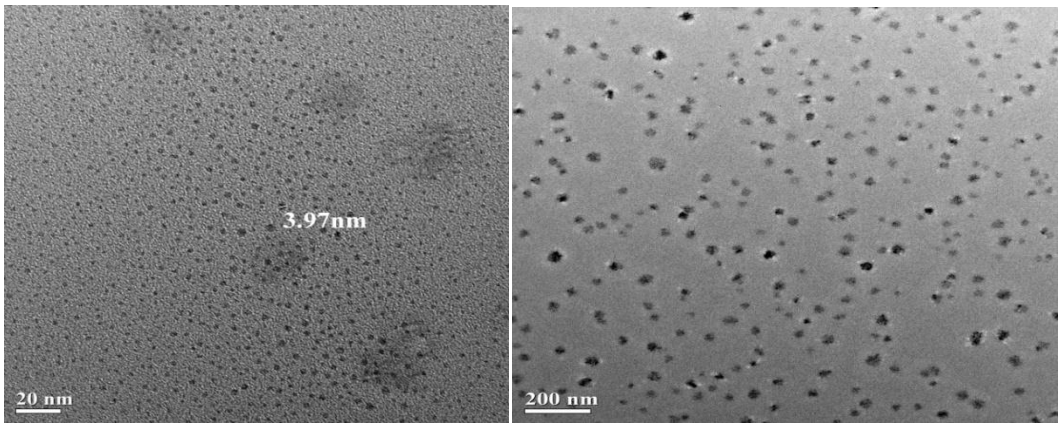


Fig 9 :- Images of TEM (Transission Electron Microscopy)

After performed Transmission electron microscopy particle was determined in 211.15 nm,148.63 nm and apart from this more smaller number of particle also detected which is 3.97 and 46.65 nm. Particle was disributed well and found as spherical shape**,** which is shown in figure no.9

Scanning Electron Microscopy:-

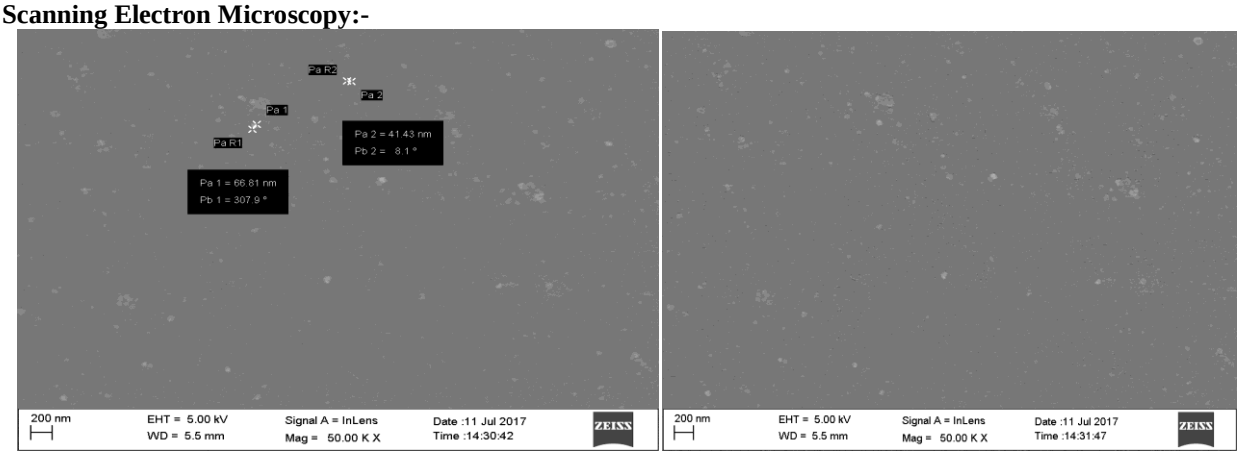


Fig:- 10 Images of SEM (Scanning Electron Microscopy)

In case of the SEM particle are nano size and distributed. Particle are found as spherical in size**.**

***In-vitro* drug released:-**

The *in-vitro* release of Ibuprofen from the Nanoemulsion gel was varied in amount according to concentration of emulsifying agents used on formulations. It is shown that the drug released in 76.93 to 91.07 % after the intervals of 300 minute for formulation F1 F2 F3 and F4 it found as 91.07%, which state as good drug released in ibuprofen nanoemulsion based gel.

Table 11:- *In-vitro* release of Ibuprofen

Time	**% of Cummulative drug released**		
	F1	**F2**	**F3**
0	0	0	0
30	10.38	1.77	9.95
60	15	8.311	19.10
90	26	13.21	27.27
120	26.99	18.67	38.79
150	29.59	22.17	49.79
180	30.90	28.98	57.02
210	35.33	30.72	68.2

240	54.96	53.98	79.5
270	62.78	58.68	85
300	80.1	76.93	91.07

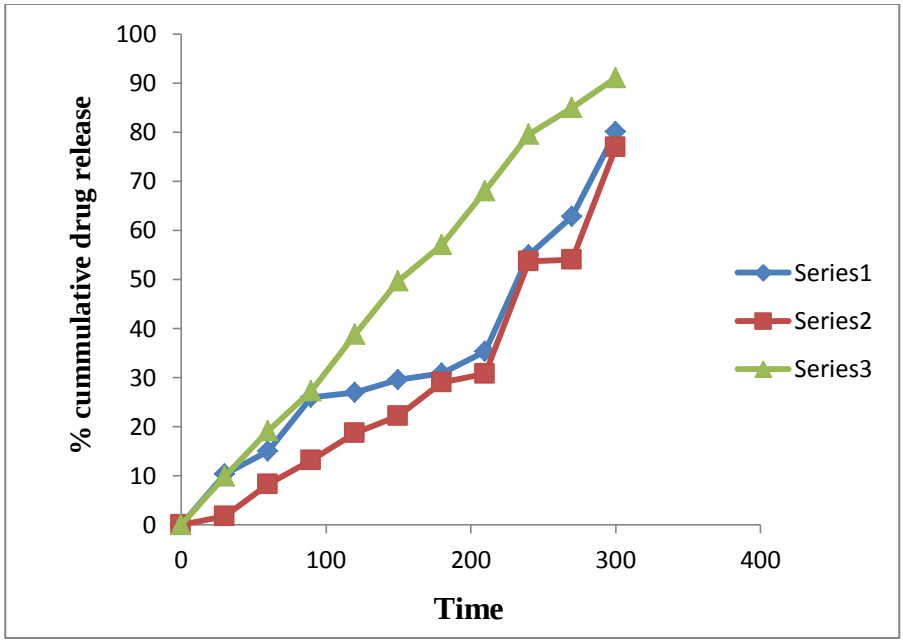


Fig 11:- % cummulative drug release from different formulations

In-vitro release of flavanoid:

Table 11:- *In-vitro* release of flavanoid

Time	**Cummulative drug released in %**		
	F1	**F2**	**F3**
0	0	0	0
30	3.04	2.22	2.54
60	12.19	10.56	12.91
90	15.28	13.52	14.87
120	30.52	29.21	30.58
150	36.69	34.59	37.98
180	39.97	36.69	33.25
210	51.96	50.54	50.29
240	58.11	55.98	56.61
270	76.41	74.94	79.65
300	85.63	81.93	82.96

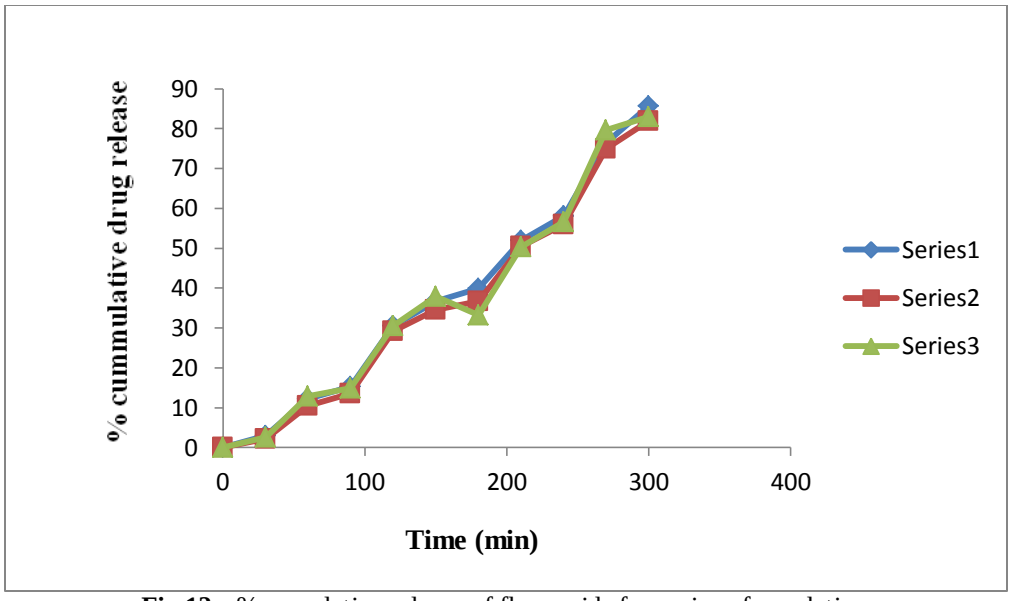


Fig 12:- % cumulative release of flavonoids for various formulation

Development of frostbite injury and animal models:-

Skin Irritation Test:-

After 24 hrs of application of novel nanoemulsion there is no erythema and edema which shows that there is no skin irritation.

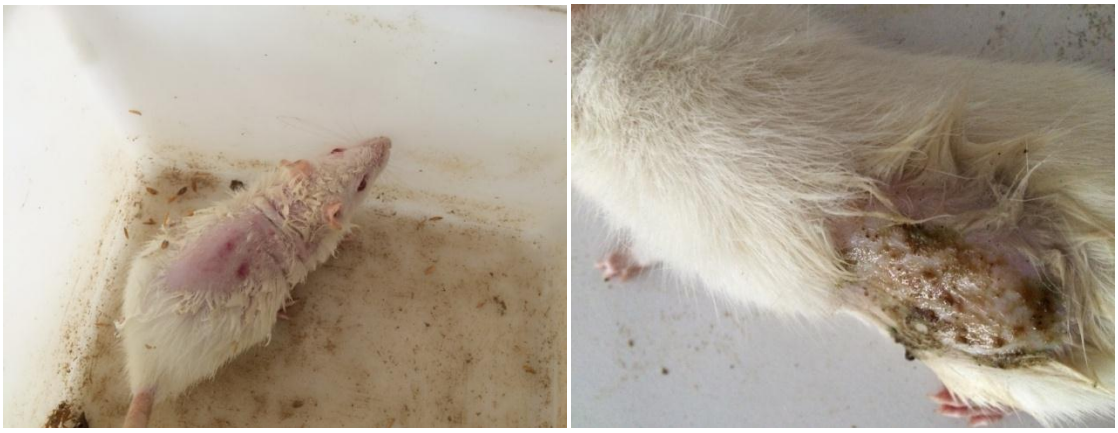


Fig 13:- Skin irritation test of rat skin

Developments of frostbite:-

Fig 14:- Development of frostbite in rat skin

Anima was treated with frozen condition and frostbite was developed over the skin of the rat as mentioned earlier. In initial stages of frostbite skin causes inflammation which is treated with nanoemulsion gel after intervals of 6 hours topically for couple of weeks and kept for observation. When Nanoemulsion used over skin Inflammation is showing decreasing after few applications. Due to the small particle size nanoemulsion formulation permeated through skin and shows good drug release.

Conclusion:-

Nanoemulsions are attractive system for the use in the cosmetic, pharmaceuticals foods and pharmaceutical industries because of amount of very less surfactant, high stability against the coalescence, lower of toxicity or irritants characteristic. Due to their better permeability characteristic through skin the colloidal dispersions of solid nanoscale particulates have received the considerable attention. Moreover, the development of high throughout production make the potential for wide spread commercial used of nanoemulsion in consumer products and medical applications. In future, we can predict that nanoemulsion will become as ubiquitous as many polymer solution and solid particulate dispersions are today.

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Declaration:-

The authors declared no conflict of interest.

Author contribution:-

The authors meet the following conditions:

- Authors made valuable contributions to design, and/or acquisition of data, and/or analysis and interpretation of data.
- Authors also participated in drafting the article and revised it extensionally for its appropriate formatting.

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