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RESEARCH ARTICLE

TOPICAL CO-DELIVERY OF INDOMETHACIN AND *NIGELLA SATIVA L.* ESSENTIAL OIL IN POLY- ϵ -CAPROLACTONE NANOPARTICLES: *IN VIVO* STUDY OF ANTI-INFLAMMATORY ACTIVITY.

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Abstract

Indomethacin is a potent, nonselective Non-steroidal Anti-inflammatory Drug (NSAID) but its low water-solubility precludes its use as topical dosage form. As with other NSAIDs, the systemic delivery is associated with high risk of serious gastrointestinal adverse events including bleeding, ulceration and perforation of stomach and intestines. Here we demonstrate a safer way of administration i.e via topical demonstrating synergistic effects when co-delivered with *Nigella sativa L.* seeds essential oil (NSSEO) in the form of co-encapsulated particles (~200 nm) of poly- ϵ -caprolactone. The particles showed penetrability across stratum corneum to dermis layer in *ex-vivo* human skin. Further study in the xylene-induced ear edema in mice was performed, and co-encapsulated particles demonstrated highest anti-inflammatory effect compared to indomethacin particles and indomethacin gels. Despite slower onset compared to indomethacin gels, the inflamed ear continued to show reduction in thickness over 8 hours of observation demonstrating synergistic and pro-longed effect contributed by NSSEO. In immunohistochemistry study of CD45+, the mice ears treated with co-encapsulated particles showed considerable reduction in lesions, epidermal-dermal separation and inflammatory cells (lymphocytes and neutrophils) infiltration as compared to other formulation. Based on microscopic evaluation, the anti-inflammatory inhibition effect of co-encapsulated particles is the highest (90%) followed by indomethacin particles (79%) and indomethacin gel (49%). The findings suggest not only skin permeability of indomethacin significantly improved but also the therapeutic effects, all provided by the presence of NSSEO in the particles. This study paves the way to

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more co-encapsulation of any other contemporary medicines in combination with this wholesome natural oil, NSSEO.

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Introduction:-

Acute inflammation is the first reaction that is identified through the increase in plasma movement and indigenous immune cells such as neutrophils and macrophages, from the blood into the damaged tissues (Ferrero-Miliani et al., 2007) (Medzhitov, 2008). Indeed, inflammation inducers cause cell membranes phospholipase A₂ activation that would trigger the release of arachidonic acid and inflammatory mediators (cytokines, serotonin, histamine, prostaglandin and leukotrienes), which facilitate leukocytes migration to the inflammation site (Sarkhel, 2016). The release of products such as histamine, bradykinin, serotonin, and cyclooxygenase (COX) is linked with the first phase of inflammation (0-1 h), whereas prostaglandins release, oxygen-derived free radicals production and polymorphonuclear leukocytes (PMN) infiltration is related to the late phase of oedema (Sadeghi et al., 2014). Globally, non-steroidal anti-inflammatory drugs (NSAIDs) are one of the mostly prescribed drugs classes. Indomethacin, being one of the NSAIDs first line non-opioid drugs prescribed for cancer pain. In addition, indomethacin is poorly water soluble (~ 0.01 mg/ml) and is only soluble at basic pH of 12 at which the drug significantly hydrolysis takes place (Lin et al., 1994). The intrinsic solubility of indomethacin was determined to be 8.8 µg/mL at pH of 9 (Comer et al., 2014). Thus, indomethacin poor skin bioavailability can be attributed to its insolubility in water for the skin pH. Various novel dosage forms (liposomes, nanospheres, nanoparticles etc.) have been formulated using indomethacin as the model drug to tackle its skin permeability problem but none of the studies use essential oil as means to increase permeability or penetrability via topical delivery. Here, we demonstrated the functional use of our selected essential oil, namely *Nigella sativa L.* seeds essential oil (NSSEO). We have observed at least two main functions this most studied oil provided in our study, namely as permeation enhancer and as efficacy modulator, both gave rise to synergistic effects. The Stratum Corneum (SC) acts as an obstacle towards efficient use of transdermal drug delivery systems development because of its low drug permeability. Therefore, numerous physical (sonophoresis, *microneedles*, and *iontophoresis*) and chemical [dimethyl sulphoxide (DMSO), ethanol, and Laurocapram (Azone[®])] skin penetration enhancement methods had been studied in order to mitigate this challenge (Benson, 2005) (Li et al., 2005). However, most of these methods cause skin irritation and damage, and ultimately disturbance of skin barrier function. Additionally, previous studies formulated indomethacin in various dosage forms like gels, cream, microparticles, nanospheres and nanoparticles. All these formulations utilized certain types of additives, either as co-solvent [propylene glycol (PG)] (Pinheiro et al., 2015), co-polymer [e.g. methoxy poly(ethylene glycol) in poly(ε-caprolactone)] (Kim et al., 2001), co-solubiliser (e.g. hydroxypropyl-β-cyclodextrin, methylcellulose) or as agent to improve encapsulation efficiency (Nagai et al., 2015). However all of the additives are lacking of evidence that can recognize them as agents that contribute to therapeutic efficacy unlike natural oil, NSSEO.

There are other studies that have also incorporated an ingredient to enhance therapeutic effect of indomethacin notably copper (Yassin et al., 2015). However, the enhanced inflammatory effects as observed in the copper-indomethacin topical delivery were thought to be attributed to the activation of copper-dependent opioid receptor. Any activation of opioid receptor will concomitantly increase risk of opioid-associated adverse effects such as sedation, dizziness, tolerance and respiratory depression.

Therefore, our main approach here was to select an agent that can confer modulation on skin permeability and penetrability of indomethacin while carrying other beneficial therapeutic efficacy. In our case, we had selected the ancient herb, most widely and thoroughly researched folklore and prophetic medicine i.e *Nigella Sativa L.* Seeds Essential Oil (NSSEO) as the co-therapeutic agent to be co-encapsulated with indomethacin in biodegradable poly-ε-caprolactone. Therapeutic efficacy of this ancient oil had been

stated in old medicinal manuscripts and its fame transcends any nation, religion and borders. NSSEO has been used for the treatment of different diseases namely rheumatism, bronchitis, asthma and others for centuries. NSSEO efficacy may be due to its biological activities such as anti-inflammatory, analgesic, immunomodulatory, spasmolytic, and anti-oxidative properties (Ahmad et al., 2013). The principal phytochemicals of NSSEO are thymoquinone, p-cymene, α -pinene, thymohydroquinone, dithymoquinone and nigellone (Al Juhaimi et al., 2013). In fact, thymoquinone formed the main part of NSSEO to which mostly NSSEO therapeutic usages are attributed to it (Ravindran et al., 2010). It is reported that the production of inflammatory cytokines can be inhibited by thymoquinone in NSSEO (Ahmad et al., 2013). The uniqueness of this study in comparison to previous researches are: (a) the usage of xylene (topical application) instead of carrageenan (injection administration route), (b) anti-inflammatory activity study via evaluation and assessment of several parameters together such as mice ears thickness, weight, histology and immunohistochemistry (IHC), (c) obtaining of information from the combination of microscopy and IHC data that help to indicate overall tissue condition, (d) employing of nanoparticles as skin penetration non-invasive enhancement approach lead to maintenance of the skin's normal function as opposed to other physical and chemical techniques. Furthermore, in this research, the mouse was used as model, which is a well-known and well described model in the literatures. Moreover, male mouse was used that can avoid eventually the interference with hormonal cycle of female mice. Here in this work, edema was shown as the increase in mice ears thickness and weight that are consecutively compared with corresponding values pre-application.

The focus of this study is in the comparative evaluation of *in-vivo* anti-inflammatory activity of nanoparticles co-encapsulated with indomethacin and NSSEO and nanoparticles containing indomethacin alone using acute cutaneous mice inflammation model. Substantially, our data found that the co-encapsulation of indomethacin with NSSEO enhanced anti-inflammatory effect via topical treatment and suggest a possible decrease in indomethacin systemic concentration that can consequently reduce the indomethacin side effects.

Materials and methods:-

Materials:-

Nigella Sativa L. Seeds Essential Oil (NSSEO) was kindly provided by the Faculty of Sciences, Ibn Zohr University, Agadir, Morocco. Poly(ϵ -caprolactone) (PCL), polyvinyl alcohol (PVA) and polysorbate 80 (Tween[®] 80) were supplied by Sigma-Aldrich, Germany. Indomethacin was provided by George Van Waters and Nat Rogers laboratory products distributor (VWR) and acetone was purchased from Laurylab, France. Electronic balance, digital micrometer (model J15, BLET), isoflurane, triethanolamine (TEA), polyethylene glycol (PEG 300), polyvinyl pyrrolidone (PVP), Carbopol ETD 2001 (C2001), and Hexylene glycol (HG) were provided by one of the common chemical products suppliers in Europe.

Preparation of nanoparticles:-

Nanoparticles containing indomethacin and NSSEO were prepared in two separate phases by nanoprecipitation technique that was firstly designed by Fessi et al, (Fessi et al., 1989). To prepare the organic phase, 200 mg PCL was dissolved in 25 ml acetone under mild heat and magnetic stirring. Then 40 mg indomethacin and 300 mg NSSEO were added into the solution of PCL in acetone for co-encapsulation of indomethacin and NSSEO. For the aqueous phase preparation, 50 mg PVA was dissolved in 50 ml Milli-Q water at mild heat and mixed with 135 mg Tween-80[®] using magnetic agitation. Subsequently, the organic phase containing NSSEO, indomethacin, PCL and acetone was added dropwise by KDS 100 Legacy Single Syringe Infusion Pump operating at 220 volts alternating current (VAC) to the aqueous phase (Figure 1). Acetone evaporation was performed afterwards (Buchi Rotavapor R-124[®]) (under reduced pressure and high temperature conditions). Mostly the parameters of nanoparticles preparation process and formulation were inspired by a systematic study which was done under the same condition by Badri et al, (Badri et al., 2017). The composition and operating condition of blank and indomethacin nanoparticles are further shown in **Table 1**.

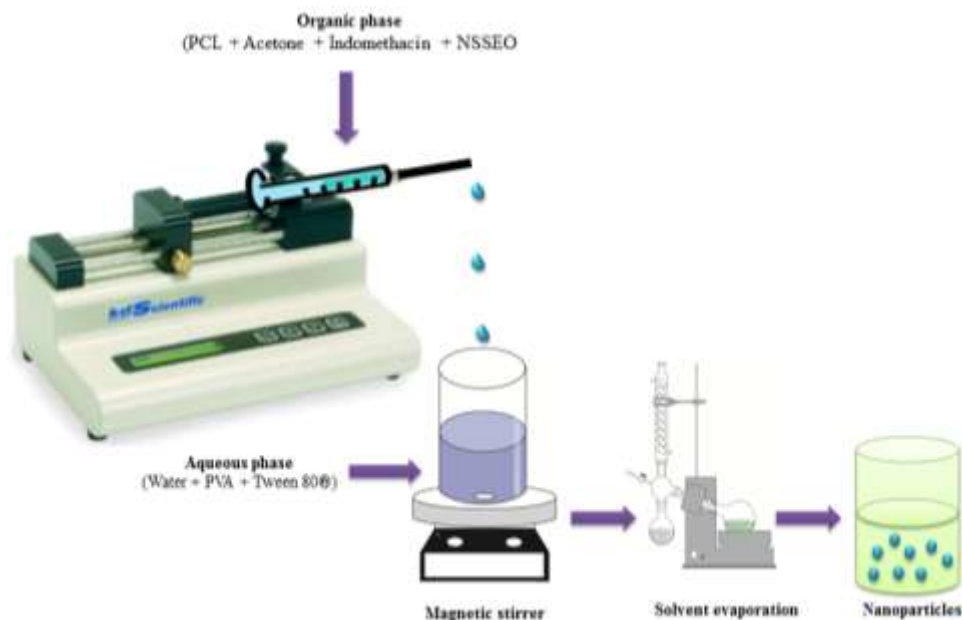


Figure1:-Schematic diagram of nanoparticles preparation by nanoprecipitation method.

Indomethacin gel preparation:-

To prepare 1% w/w indomethacin gel, 1% w/w of gel forming agent, C2001, was slowly added in small increments into a vortex of 57 % w/w sterile water under continuous magnetic agitation. Then, resin was added to prevent the entrapment of air, and stirring was continued at a reduced speed. The resulting dispersion was stored at rest mode in the dark for 24 h to obtain a homogenous solution. HG (30 % w/w) and PEG 300(10 % w/w) as solvents, were first mixed together and then added into the 1 % w/w indomethacin to dissolve indomethacin. The solution was then poured into the mixture in small portions with constant stirring until homogeneity was achieved. Afterwards, 1 % w/w of TEA as neutralizer was added to the mixture in order to increase the pH and trigger the formation of gel. The obtained gel packaging was carried out in amber glass containers and stored for 24 h in a dark place at room temperature ($20 \pm 2^\circ\text{C}$) (Shawesh et al., 2003).

Table 1:-Nanoparticles composition and operating condition

(a). PCL based blank nanoparticles

Organic Phase		Aqueous phase			Operating condition	
PCL concentration (mg/ml)	Acetone volume (ml)	Tween@ 80 concentration (mg/ml)	Milli-Q water volume (ml)	PVA concentration (mg/ml)	Stirrer speed (rpm)	Organic phase injection rate (ml/min)
8	25	2.7	50	1	300	9

(b). PCL based nanoparticles loaded with indomethacin

Organic Phase			Aqueous phase			Operating condition	
PCL concentration (mg/ml)	Acetone volume (ml)	Indomethacin concentration (mg/ml)	Tween@ 80 concentration (mg/ml)	Milli-Q water volume (ml)	PVA concentration (mg/ml)	Stirrer speed (rpm)	Organic phase injection rate (ml/min)
8	25	1.6	2.7	50	1	300	9

(c). PCL based nanoparticles containing indomethacin and *Nigella Sativa* L. Seeds Essential Oil

Organic Phase				Aqueous phase			Operating condition	
PCL concentration (mg/ml)	Acetone volume (ml)	Indomethacin concentration (mg/ml)	NSSEO concentration (mg/ml)	Tween® 80 concentration (mg/ml)	Milli-Q water volume (ml)	PVA concentration (mg/ml)	Stirrer speed (rpm)	Organic phase injection rate (ml/min)
8	25	1.6	6	2.7	50	1	300	9

Nanoparticles characterization:-

To characterize the prepared nanoparticles, their size, zeta potential, morphology, and encapsulation efficiency studies were carried out.

Particle size and zeta potential:-

To measure nanoparticles size and zeta potential, Malvern particle size analyzer using dynamic light scattering (Zetasizer - Nano ZS, Malvern instruments limited, UK) was used. Prepared nanoparticles were dispersed in 1mM NaCl solution previous to each zeta potential measurement whereas nanoparticles size measurement was carried out after colloidal dispersion dilution within a 1 mL of distilled water. All measurements were taken place in triplicate at room temperature (25 °C) and their average was taken as the result.

Nanoparticles morphology:-

For nanoparticles shape and appearance assessment, Transmission Electron Microscopy (TEM) was employed. Nanoparticles TEM has been taken place by Philips CM-120 Transmission electron microscope (CMEABG, Claude Bernard University Lyon 1, France) by 120 kV accelerating voltage. To this end, a drop of nanoparticles suspension was diluted in 2 ml of *Milli-Q water* by micropipette and consecutively one drop of this dilution was placed on the carbon-coated copper grid. Supplementary amount of nanoparticles suspension was removed via blotting the grid through filter paper and instilled nanoparticles suspension on the grid dried prior to TEM analysis at room temperature.

Ex vivo skin penetration:-

Skin penetration study was carried out on the *ex vivo* human skin model by confocal laser scanning microscopy (CLSM). Indeed, CLSM is a non-invasive optical imaging technique. The distribution of applied nanoparticles that contain active ingredient and fluorescent agent (Core Shell Evidots) in skin can be visualized and inspected by CLSM (Pygall et al., 2007) (Alvarez-Román et al., 2004). Fresh excised human abdominal skin was obtained from plastic surgery. Samples for this study comprised of: (a) mixture of 25 µl Core Shell Evidots (CSE) and 50 ml distilled water liquids, (b) poly(ε-caprolactone) based NPs loaded with indomethacin and CSE, (c) poly(ε-caprolactone) based NPs loaded with indomethacin, CSE and NSSEO (preparation of samples b, and c described in Table 1, except that for *ex vivo* study, 50 µl CSE were added into the organic phase). Afterwards prepared formulations were topically applied at room temperature on a defined area of skin (100 µL/cm²) following a gentle massage for 30 seconds. A skin area remained untreated and served as control. After 1 h of CSE and NPs application, a 3 mm punch biopsy specimen was taken from each area. Skin biopsies were placed at - 80 °C until use for the study. The frozen skin samples were embedded in Tissue-Tek® and cut in 7 µm vertical cryostat sections. Sections were mounted using VECTASHIELD® mounting media and were imaged under a CLSM (Leica SP2 AOBS microscope with blue laser excitation at 405 nm, 63x oil immersion objective). Blue fluorescence intensity was the indicator of NPs penetration assessment through the skin in a comparative manner between treated and untreated (control) human skins.

In vivo* anti-inflammatory activity study:-*Animals:-**

Adult male Swiss mice (6 - 8 weeks age) were housed and given diet at libitum under standard laboratory conditions of temperature, and relative humidity. On the day of the experiment, mice were individually weighed and tagged with a temporary tail marking. For each step of experiment, mice were anesthetized using isoflurane 2.5 %, with 1 L/min flow for induction, then isoflurane 1.5%, with 0.5 L/min flow for maintaining anesthesia. In this study, four groups of animals were used (n = 7 each) in accordance to the minimum number for a proper statistical analysis. Regular observation of the animals during the habituation period was performed. Experiments were in compliance with guidelines for study in animals' laboratory and were approved by the Ethics Committee of Animal Experimentation of French National Center for Scientific Research (CNRS).

Acute ear edema induction:-

In order to induce an acute edema in mice ears, xylene as a phlogistic agent was topically applied on the inner and outer surface of the right ear of mice (30 μ L/ear). The left ear was considered as negative control and received only distilled water (20 μ L/ear).

Application of designed formulations:-

To apply formulations separately and to study the anti-inflammatory effect of applied formulations comparatively, animals were randomly assigned into four groups (Figure2). **Group I** animals have employed to evaluate the absence of anti-inflammatory activity of the blank nanoparticles (20 μ L/ear). **Group II** animals were used to assess the anti-inflammatory activity of the nanoparticles containing indomethacin (20 μ L/ear). **Group III** animals have used to evaluate the anti-inflammatory activity of nanoparticles containing indomethacin and NSSEO (20 μ L/ear). **Group VI** animals were used as a positive control, and 1% indomethacin gel (100 mg/ear) was applied. The quantity of indomethacin in all indomethacin-containing formulations is constant (1 mg/ear), following Garrido et al, (Garrido et al., 2004). In addition, NSSEO quantity in the formulation that contained NSSEO was 2 mg/ear. The left ear of mice within all four groups was considered as negative control on which the distilled water (20 μ L/ear) was applied. Furthermore, formulations have topically applied after application of xylene on the inner and outer surface of the right ear of all four groups of mice.

Anti-inflammatory activity assessment:-

Experiments were carried out based on Oliveira et al, method (Liduína Maia de Oliveira et al., 2013). The thickness (μ m) of each ear was measured using a precise digital micrometer (model J15, BLET) that was put close the ear tip, just distal to the cartilaginous ridges. Measurement was performed prior to the application of xylene (0h) and then at 1h, 2h, 3h, 6h, and 8h time intervals after induction of an inflammatory response. The edema is evaluated based on the alteration of the thickness of mice right ear versus mice left ear. Anti-inflammatory activity of prepared formulations was assessed by comparing weight (mg) and thickness (μ m) reduction with respect to the positive control and pre-injection values (Figure 7).

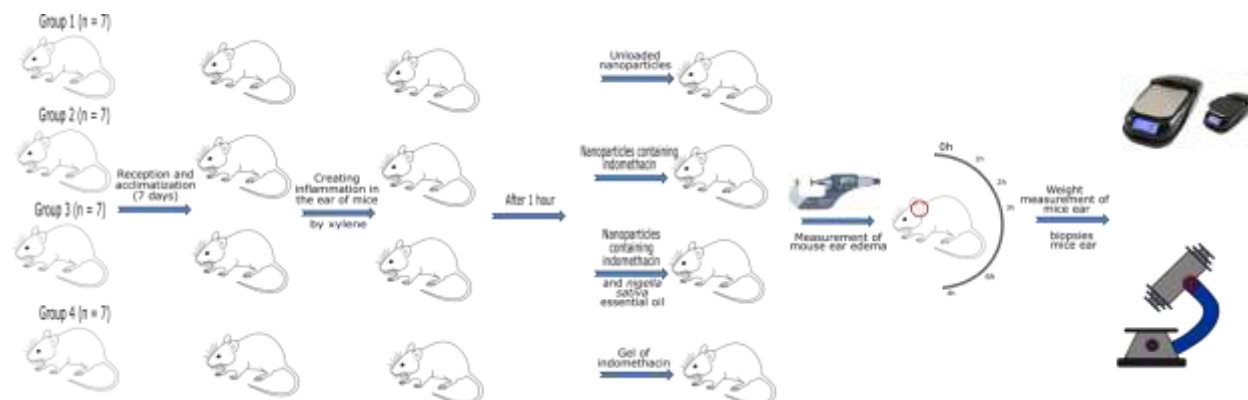


Figure 2:-*In vivo* study experimental design scheme, Four Groups (n = 7 each) of mice were employed, (Group 1 for anti-inflammatory activity assessment of the blank nanoparticles, Group 2 for evaluation of anti-inflammatory activity of nanoparticles containing indomethacin, Group 3 for the study of anti-inflammatory activity of nanoparticles containing indomethacin and NSSEO and Group 4 were used as a positive control, to investigate the anti-inflammatory activity of 1% indomethacin gel).

Histology analysis:-

Mice removed ears was studied from two aspects of simple histology and immunohistochemistry analysis. The induction of skin inflammation can take place in 1 – 2 h of exposure, which is described via increased blood circulation, vascular permeability, infiltration of leukocyte into the skin, degeneration of epidermis, boosted oxidative species levels, and DNA damage.

Mice were first euthanized and ears were cut and fixed (10 % formaldehyde for 24 h- 48 h) and processed by standard methods of histology. Afterwards, fixed mice ears were embedded in paraffin for 3 h, sliced into 3 μ m sections and stained by Hematoxylin-Phloxine. Mice ears process was performed in Leica ASP300 S. The samples preparation for simple histology microscopy observation and immunohistochemistry (IHC) followed the same procedure up to cutting of mice ears (1 single piece). Subsequently, samples were mounted on the Superfrost[®] gelatin-coated slides (SIGMA – ALDRICH). On the other hand, IHC Tissue prepared samples were mounted on the Superfrost[®] slides that was then coated with water, to remove background noise gelatin wasn't used in IHC. Tissues were observed by Axio Scan Z1 de Zeiss slide scanner at a magnification of 20X.

Immunohistochemistry (IHC) study:-

Primary monoclonal mouse antibodies (streptavidin peroxidase conjugates) raised against CD45 (clone PD7/26) was used to analyse leucocytes. Embedded tissues was first treated with Cell Conditioning Solution (CC2) before paraffin was removed using the absolute ethanol. Inflammation was detected using a biotinylated anti-mouse rabbit IgG secondary antibody, followed by colorimetric recognition using Streptavidin-biotin peroxydase detection system (DISCOVERY DAB Map Detection Kit (RUO)). The counterstaining of sections was performed with hematoxylin (4 min) and bluing reagent (4 min), and sections were then mounted under coverslips (Figure 3) to be viewed under a slide scanner (Axio Scan Z1 de Zeiss) at 20X magnification. Obtained images were processed with Fiji software (image processing package) and analysis was performed for epidermis integrity, dermis thickness, infiltration of leukocytes and edema.

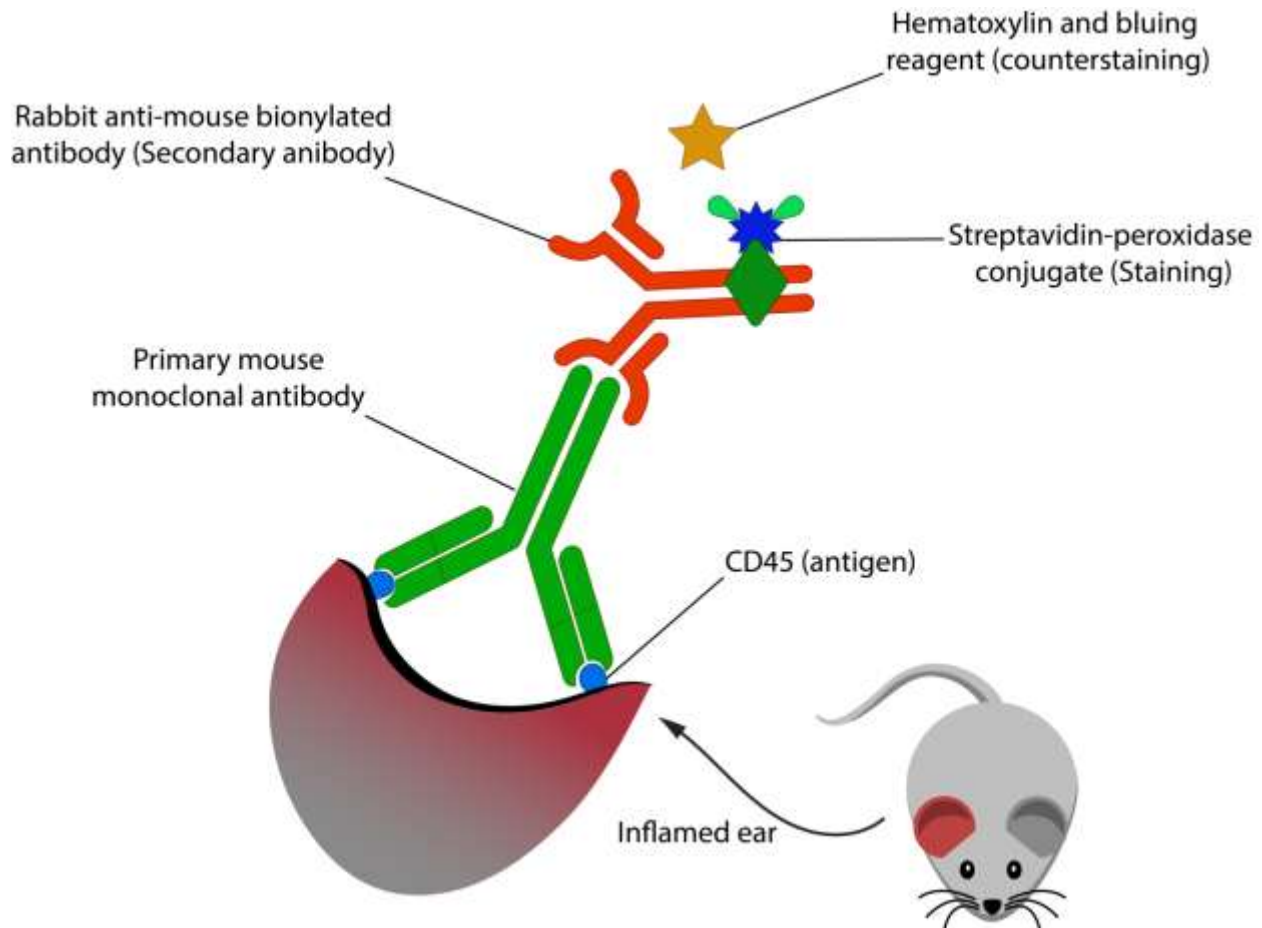


Figure 3:-Indirect enzyme linked immunohistochemistry (streptavidin peroxidase conjugated method) illustration.

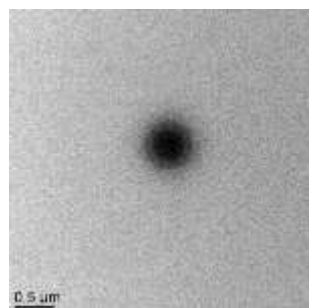
Statistical analysis:-

Data were analyzed by GraphPadPrism7.0 software and shown as mean \pm standard deviation. Multiple comparisons were carried out by one-way analysis of variance (ANOVA) at $P < 0.01$ significance.

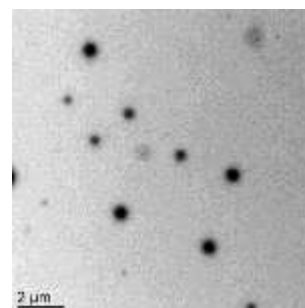
Results And Discussion:-

Nanoparticles characteristics:-

Nanoparticles size and zeta potential were ranged between $230 - 260 \pm 12.47$ nm and $(-20$ mV) up to $(-30$ mV) ± 4.082 , respectively. The pH of the colloidal dispersion was around 6 ± 0.82 , whereas the encapsulation efficiency of indomethacin and NSSEO within the designed nanoparticles was 70, 84 ± 5.73 % respectively. Prepared nanoparticles size distribution was about 0.166 ± 0.007 . Transmission Electron Microscopy (TEM) analysis showed that nanoparticles have a spherical and regular form (Figure 4).



(a) 0, 5 μ m scale bar



(b) 2 μ m scale bar

Figure4:-Transmission Electron Microscopy images of PCL based nanoparticles loaded with indomethacin and NSSEO, (a). 0.5 μm scale bar, and (b). 2 μm scale bar showing nanoparticles smooth surface and regular form.

Nanoparticles loaded indomethacin and NSSEO: are able to penetrate *ex vivo* fresh human skin:-

The skin plays principal functions such as barrier role, temperature control role and repair role that contribute to homeostasis process of human body (Sala et al., 2018). Skin has a potential application in drug delivery thanks to its large surface area. Prevention of first pass metabolism, minimization of pain and possible controlled release of drugs are from the advantages of the Topical or transdermal delivery over the conventional oral and intravenous dosage forms (Desai et al., 2010). Skin drug delivery (SDD) that is a smart method to the treatment of many diseases, cover in general dermal and transdermal drug delivery (Sala et al., 2018). Active molecules after topical application of nanoparticles can be absorbed via pathways such as transcellular, intercellular or transappendageal (Figure 5). It is possible that either topically applied nanoparticles place into the skin without degradation or with degradation nearby to the skin surface, consecutively loaded active molecule would penetrate into the layers of skin. Nanoparticles physicochemical properties including size, surface charge, used nanomaterials properties, and so on are governing the interaction of nanoparticles with skin (Desai et al., 2010).

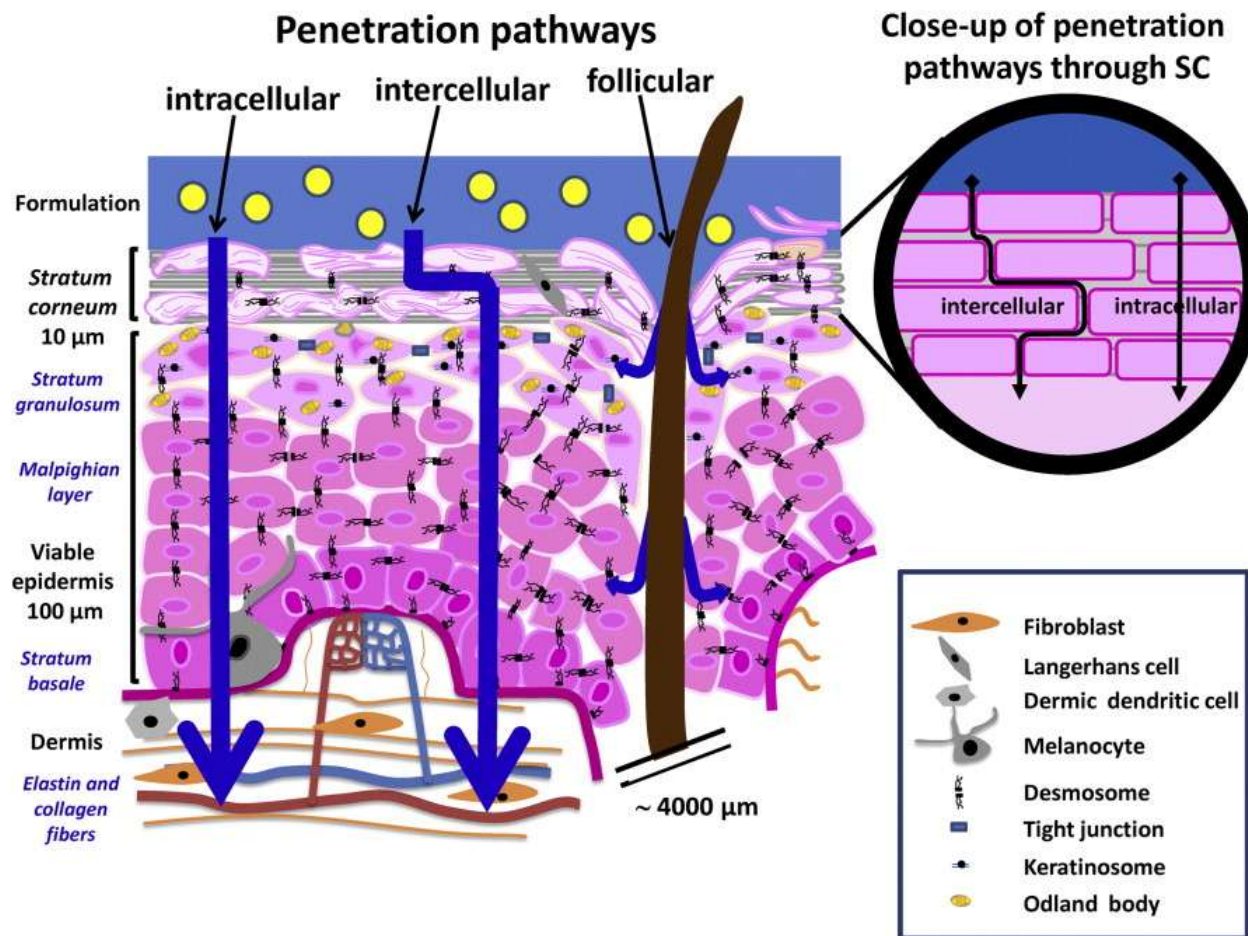


Figure 5:-Sketch of the three penetration pathways: transcellular, intercellular and follicular. The upper right inset is a close-up of the SC showing the transcellular pathway and the tortuous intercellular pathway (Bolzinger et al., 2012).

CLSM images of skin histological sections are shown in Figure 6 and Figure 7. The images taken from the control skin section demonstrated, in which an autofluorescence in the dermis can be observed coming from collagen and elastin fibrous structures. The image obtained from sample B (Fig. 6) showed the total

fluorescence comprised of the autofluorescence and the fluorescence of the CSE. For samples Fig. 6B and Fig. 6C, fluorescence was distributed throughout the *Stratum Corneum* (SC) and the dermis. For sample Fig. 6D, fluorescence was observed in the epidermis and dermis, fluorescence signal of the dermis appeared to be highest compared to control. The Figure C illustrated skin location of NPs, which are visible only through the epidermis. The fluorescence labeling by CSE confirmed that NPs could be observed using CLSM. Fluorescence emission provided semi-quantitative information on the skin penetration of NPs. CLSM images proved that NPs would penetrate the skin, reaching the dermis. As no fluorescence was observed in the SC after application of NPs with *Nigella Sativa L* Seeds Oil (Figure 7D), the relative accumulation of fluorescence in the *stratum corneum* after application of NPs without NSO as can be seen in Figure 6B indicate that NSO facilitates the penetration of NPs throughout the outermost layer of the epidermis. Nanoparticles were visualized across the epidermis but were hardly detectable in the dermis because of the autofluorescence of collagen and elastin fibers. These findings draw a conclusion that nanoparticles loaded with indomethacin and NSSEO can penetrate the SC barrier to improve the anti-inflammatory activity of indomethacin.

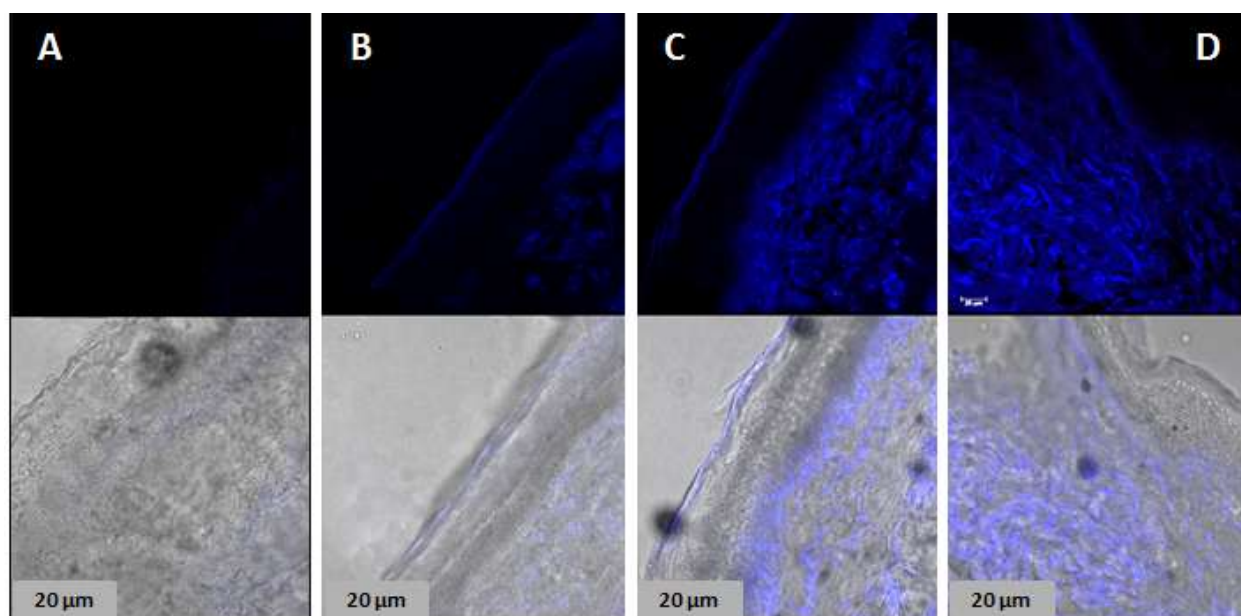


Figure 6:- CLSM images of NPs deposited on human skin. Scale bars are 20 µm. The CSE used in this study have an emission peak at 516 nm. Scale bars are 20µm, (A): control or untreated skin with nanoparticles, (B): mixture of 25 CSE and distilled water, (C): nanoparticles loaded with indomethacin and CSE, and (D): nanoparticles loaded with indomethacin, NSSEO and CSE.

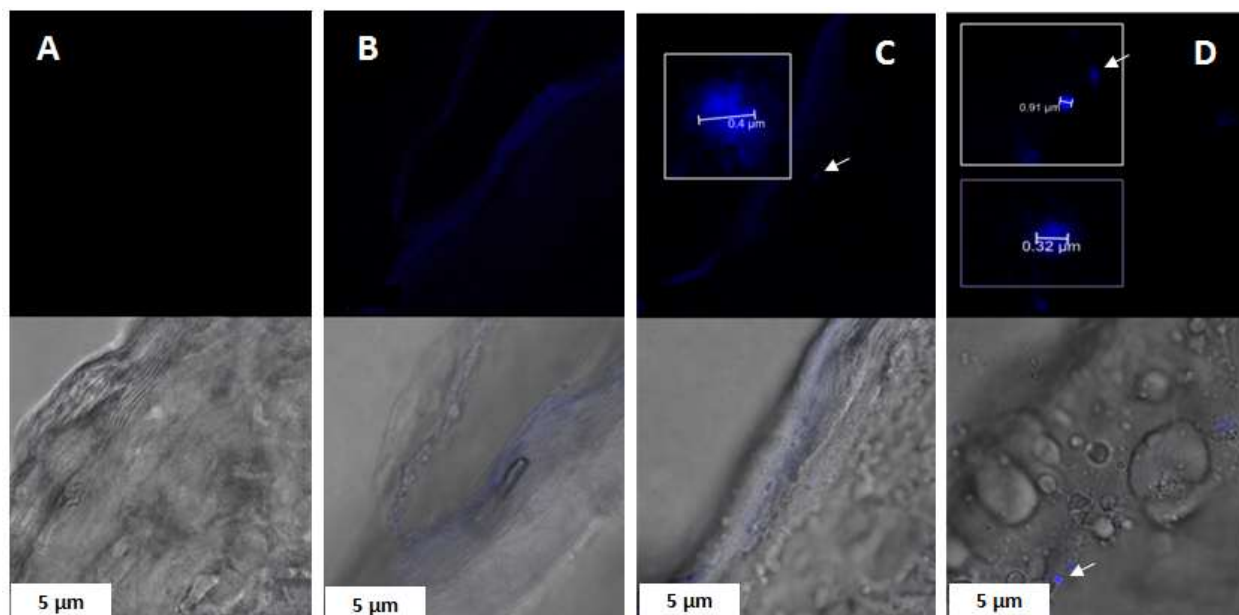


Figure7:-CLSM images of NPs (arrow) through the epidermis, Scale bars are 5 μ m. (A): control or untreated skin with colloidal dispersion, (B): mixture of 25 CSE and distilled water, (C): nanoparticles loaded with indomethacin and CSE, and (D): nanoparticles loaded with indomethacin, NSSEO and CSE.

Since *ex-vivo* investigation results of this research were promising therefore it let us to go further and perform the *in-vivo* anti-inflammatory study as well.

Nanoparticles containing indomethacin and NSSEO: has higher anti-inflammatory efficacy:-

As can be seen in Figure 8, upon the application of all four formulations at the beginning of experiment (0 h), no significant differences were observed in mice ears thickness. This can be explained by the SC permeability barrier property towards xylene, which was applied for the creation of edema (thickness). However, in 1st hour the thickness decreased significantly by indomethacin gel treatment while the thickness was stable for three other formulations. Since polymer based nanoparticles and gel are two different forms therefore this can be indicated to the imprisoning of indomethacin and NSSEO within the polymeric nanoparticles that is not the case for indomethacin gel ($P < 0.01$) in other words release of indomethacin from nanoparticles take time to exert its anti-inflammatory activity. In addition, in 2nd hour the thickness of mice ears by nanoparticles containing indomethacin and NSSEO in comparison with the blank nanoparticles was significantly reduced as compared to the indomethacin gel ($P < 0.001$). On the other hand, in the 3rd hour, thickness depression by nanoparticles loaded with indomethacin and NSSEO was highly significant in comparison to indomethacin gel ($P < 0.0001$). Furthermore, in 6th and 8th hours the thickness reduction by nanoparticles loaded with indomethacin and NSSEO together, nanoparticles loaded with indomethacin alone and indomethacin gel was respectively significant ($P < 0.00001$). This inflammatory inhibition of nanoparticles containing indomethacin and NSSEO reached its peak 8 h after xylene application and showed the highest superiority in reducing mice ear thickness.

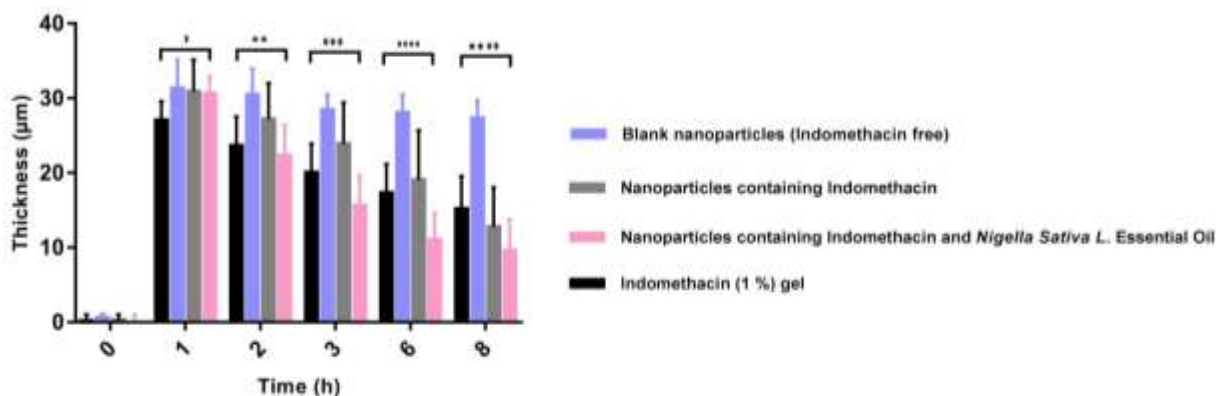
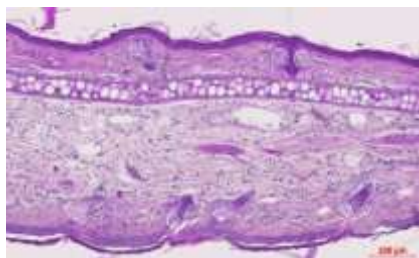


Figure 8:-Anti-inflammatory activity assessment is based on mice ear thickness measurement in 0h, 1h, 2h, 3h, 6h, and 8h time intervals. Mice ears thickness reduction by all four formulations is indicated by bars. Data described in Figure 8 shows that nanoparticle loaded with indomethacin and NSSEO has the highest anti-inflammatory activity among these formulations except 0h due to NSSEO enhancing effect. $P < 0.01$ is noted *, $P < 0.001$ is noted **, $P < 0.0001$ noted *** and $P < 0.00001$ noted ****. Multiple comparisons were carried out by one-way analysis of variance (ANOVA) at $P < 0.01$ significance.

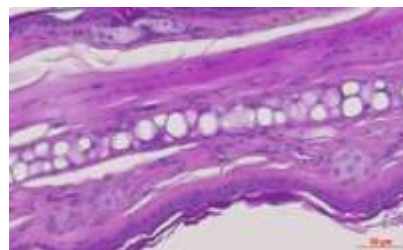
Xylene as phlogistic agent endorses neurogenic inflammation by acting on immune cells, mast cells, and vascular smooth muscle (Liduína Maia de Oliveira et al., 2013). The left ear of mice within all four groups was considered as negative control on which the distilled water (20 µl/ear) was applied. The experiments performing intervals were limited to the 8 h.

Tissue inflammation histopathological changes can be dealt with easier via nanoparticles loaded with indomethacin and NSSEO:-

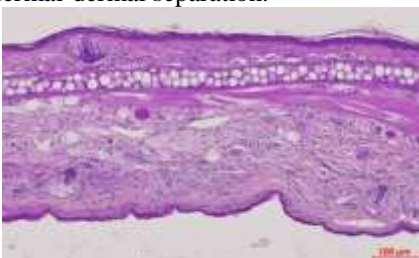
Histology and immunohistochemistry studies have shown that subsequent to the xylene contact, rat skin histopathological changes consist of epidermal-dermal layers separation and infiltration of granulocyte into the skin at 4 h and 6 h time points (Figure 9; Figure10).



(a). Xylene induced mice ear edema treated with blank nanoparticles denoting more lesions, epidermal-dermal separation.



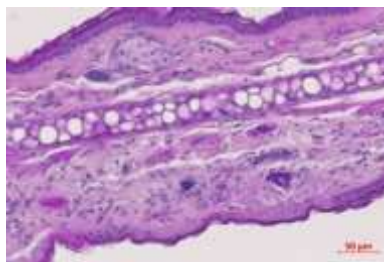
(b). Xylene induced mice ear edema treated with distilled water (negative control) showing normal cell histology structure.



(c). Xylene induced mice ear edema treated with nanoparticles loaded with indomethacin.



(d). Xylene induced mice ear edema treated with nanoparticles loaded with indomethacin and *Nigella Sativa L.* Essential oil, showing remarkable amelioration of the lesions, epidermal-dermal separation, and inflammatory cells.



(e). Mice ear treated with indomethacin gel (positive control).

Figure 9:- Histology images of mice ear with a magnification of 20X, Scale bars represent for (a), (c), (d) 100 µm and for, (b) and, (e) 50 µm. It shows that epidermal-dermal layers separation and infiltration of granulocyte into the skin reduced by nanoparticles loaded with NSSEO and indomethacin (d) in comparison with other formulations (a), (b), (c) and (e) is prominent thanks to NSSEO presence.

The homogeneous eosinophilic substance accumulation was observed at the epidermal-dermal separation areas implying skin damage and/or inflammation associated with xylene application. Our findings in this research are in accordance with Gunasekar et al, in 2003 (Gunasekar et al., 2003). In addition, inflammatory lesions were more evident than was previously reported (Sadeghi et al., 2014). The topical application of designed formulations including indomethacin gel (1 %), nanoparticles loaded with indomethacin alone and nanoparticles loaded with indomethacin and NSSEO, could turned down the previously histological changes in different ratio. However, mice ears treated with distilled water (negative control) and mice ears treated with blank nanoparticles (indomethacin free) did not show any changes in the above mentioned histological changes. Treated mice ears nanoparticles loaded with indomethacin and NSSEO shown extravagant lesions, epidermal-dermal separation, and inflammatory cells (lymphocytes and neutrophils) infiltration reduction effect as showed in Figure 9(d).



(a). Xylene induced mice ear edema treated with distilled water (negative control).



(b). Xylene induced mice edema ear treated with blank nanoparticles.



(c). Xylene induced mice ear edema treated with nanoparticle loaded with indomethacin.



(d). Xylene induced mice ear edema treated with nanoparticles loaded with indomethacin and *Nigella Sativa* L. essential oil.



(e). Xylene induced mice ear edema treated with indomethacin (1 %) gel (positive control).

Figure 10:-Microscopic evaluation of inflammatory cell infiltration differential profile, epidermis integrity, dermis thickness, and edema in mice ears, all samples were assessed in representative areas with increased 20x.

Immunohistochemical evaluation of CD45+, cells in the inflamed and non-inflamed ears tissues of mice. Samples were taken after 9 h xylene application. The mice ear sections were stained with Hematoxylin-Phloxine and all representative tissue section slides were observed with increased. The images show the thickness of the dermis, sebaceous glands, blood vessels and leukocyte infiltration as the criteria for evaluation of anti-inflammatory activity. These criteria are most strongly decreased in (d).

As can be seen in images (Figure 10), the blue color shows the normal cells while the brown color indicates the CD45 that is directly correlated with the inflammation. The number of normal and inflamed cell nucleus were counted and provided the percentage of inflammatory inhibition. Tissue inflammation quantity was determined by the division of CD45 number on the normal cell nucleus number. The anti-inflammatory inhibition percentage of designed formulations such as blank nanoparticles, indomethacin gel (1 %), nanoparticles loaded with indomethacin, and nanoparticles loaded with indomethacin and *Nigella Sativa L.* seeds essential oil were respectively 9 %, 47 %, 79 % and 90 %.

The obtained data from this research support the concept that NSSEO would exert its anti-inflammatory activity and boost the efficacy of indomethacin, and decrease its dosage and consequent side effects.

Conclusions:-

This study firstly reports that PCL based nanoparticles loaded with NSSEO can significantly improve cutaneous penetration of indomethacin as a noninvasive approach. In another words there would not be required to irritate, or disturb skin functions for enhancing drug delivery to skin and overcoming SC barrier properties. Furthermore, this study reinforces the anti-inflammatory activity enhancement of indomethacin by NSSEO within the polymeric nanoparticles. Consequently, for providing the same efficacy by taken dose of indomethacin can be decreased due to NSSEO existence with indomethacin in the formulation in order to reduce its side effects throughout the digestive system. Indeed NSSEO anti-inflammatory is associated with presence of thymoquinone. Further researches are necessary to investigate NSSEO different amounts effects on its anti-inflammatory activity and to support NSSEO clinical applications.

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