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Development, characterization and evaluation of *in vitro* anti-inflammatory activity of *Withania coagulans* extract and extract loaded microemulsion

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Abstract: Herbal medicines are gaining importance due to more advantages and less toxic effects. *Withania coagulans* is natural plant that possesses multiple activities. Its main constituents are *withaferin* and *withanolide*. The purpose of present study is to identify main constituent of *Withania coagulans* and preparation of extract loaded micro emulsions. *Withania coagulans* fruit extract in methanol/chloroform (1:1) was collected in semisolid form and LCMS was done to identify active compound, and then micro emulsions were prepared using Tween 80: Transcutol (1:1) Frankincense oil, and water to enhance its stability for topical application. Five formulations were prepared by Pseudo ternary phase diagram and evaluated for pH, conductivity, viscosity, drug contents, particle size analysis, and polydispersity. *Withania coagulans* extract was evaluated for anti-bacterial activity against (*Staphylococcus aureus, E. coli*, and *S. typhi*) and anti-fungal activity against (*Candida albicans* and *Aspergillus niger)*. Anti-inflammatory activity was checked for both extract and Extract based micro emulsion. Among all five formulations F5 shows best physiochemical properties with small globule size, good stability and high anti-inflammatory activity. Based on these results it was concluded that *Withania coagulans* extract loaded micro emulsions can be used for topical application with promising anti-inflammatory activities. Data for *in-vivo* studies for checking the topical effect of *Withania coagulans* is provided elsewhere.

Keywords: *Withania coagulans* extract, microemulsion, anti-inflammatory, antimicrobial, anti-fungal activity.

INTRODUCTION

Withania coagulans is known as paneer Doda belongs to the family S*olanaceae* founds in Pakistan and its surroundings. *Withania coagulans* is commonly known as cheese maker (Ismail *et al*., 2017). The active constituent is *withanolide, withaferin*.

Withania. coagulans is used in tooth pain (Najeeb *et al*., 2019), Asthma and liver disorder. It possess Anti nociceptive, antimicrobial, antifungal and anti-inflammatory properties. In diabetes induced neuropathy and nephropathy (Kumar *et al*., 2017) (Ismail *et al*., 2017), as hepatoprotective agent (Qureshi *et al*., 2019). In breast cancer for its apoptotic activity (Ahmad *et al*., 2017). As immunosuppressant (Kumar and Veeranjaneyulu, 2018), as free radical scavenger and in Insomnia (Kumar and Kumar, 2019).

Micro emulsions are widely used due to their thermodynamic stability. Micro emulsion is isotropic mixture of two immiscible liquid which gives a single stable phase by the addition of surfactants (Rivera-Rangel *et al*., 2018). Micro emulsions are used for both hydrophilic and hydrophobic drugs and also for the incorporation of extracts (Radi and Abbasi, 2018). Number of drugs and extracts have been incorporated in micro emulsion because of their stability, ease in preparations, small particle size and large surface area (Zhang *et al*., 2020a). A number of drugs like piroxicam, diclofenac, ibuprofen and naproxen are widely used for their pain reliving and anti-inflammatory effects but at same time they provide skin irritation effect after topical application (Amiri-Rigi and Abbasi, 2019).

The purpose of present study was to develop *Withania coagulans* extract based micro emulsion because of its anti-inflammatory properties and also evaluation of antimicrobial and antifungal properties of extract.

MATERIALS AND METHODS

W*ithania coagulans* fruits were purchased from local market and identified. Methanol was purchased from Sigma Aldrich (Germany), Frankincense oil, was purchased from (Co-Natural company), Tween 80 was purchased from (Sigma Aldrich), Transcutol-p was provided as gift sample by (Morgan chemicals Karachi, Pakistan).

Preparation of Withania coagulans extract

Withania coagulans fruits (3kg) were obtained from local market. The outer cover was removed and the fruit part

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was used for the extraction purpose. The fruits were then soaked in (1:1) methanol and chloroform mixture 4-5 days. Maceration method was adopted. Soaked fruits were than stained using muslin cloth and the solid fruit part was removed. After that extract obtained was subjected to rotatory evaporation to remove solvent at 45°C. Then concentrated extract was subjected to drying by placing the extract in desiccator. A semi solid brown paste obtained was kept in stoppered glass container and kept in refrigerator for further studies.

EPI-MS/MS* of *Withania coagulans

EPI-MS/MS analysis of *Withania coagulans* was done in order to identify the active compound in the mixture of organic portion of *Withania coagulans*. Linear Ion Trap Mass Spectrometer (Thermo Scientific, USA) equipped with electro spray ionization (ESI) source was used. Direct insertion method was adopted with a flow rate of 9µl/min. Positive and negative mode of ionization was applied. Compound was detected within mass from 50-2000 *m/z*. Capillary voltage was kept at 4.3 kv and capillary temperature set at 285°C. Collision induced energy range varied from 20-30 (Mirjalili *et al*., 2013).

Construction of Pseudo ternary phase diagram

This Method was adopted from (Zhang *et al*., 2020b). Pseudo ternary phase diagram was constructed to find out micro emulsion region. Surfactant/co surfactant were taken in 1:1 and mixed with oil at various weight ratios 0.5:9.5, 1.0:9.0, 1.5:8.5, 2.0:8.0, 2.5:7.5, 3.0:7.0, 3.5:6.5, 4.0:6.0, 4.5:5.5, 5.0:5.0, 6.0:4.0, 7.0:3.0, 8.0:2.0, 9.0:1.0, 9.5:0.5 (w/w). Mixture was taken in 15 test tubes and 50µl water was added in each test tube after every 1-2 hr. Tubes were vortex for 2 mins. and observed for any change in physical appearance. Samples were visually analyzed against dark back ground by enlightening with white light for transparent, translucent, and turbid/milky appearance of resultant mixture. Pseudo ternary phase diagram is shown in fig. 1 below.

Preparation of Withania coagulans extract loaded micro emulsions

Micro emulsion was prepared by using surfactant mixture (Tween 80, co-surfactant Transcutol P), and Frankincense oil by pseudo ternary phase diagram. Surfactant/ co surfactant mixture was added in the Frankincense oil and water was added dropwise gradually until a clear micro emulsion region appeared. Micro emulsion region from phase diagram was noted and five formulations were selected from the micro emulsion region for loading extract into micro emulsion. Up to 10% extract was loaded in five formulations. (Percentage of extract used was in accordance with reference to the dose of the drug used for topical application in comparison to extract. Data provided else ware) Formulations were evaluated for, pH, Conductivity, Viscosity, Drug content, particle size and potential and poly dispersity. Extract and Extract based emulsion was evaluated anti-inflammatory activity. Antimicrobial and antifungal screening of extract was also done. Composition is given below in table 1.

Characterization

Physicochemical properties of micro emulsion

Viscosity measurements

The Viscosity of ME was measured by rotational Viscometer (Brookfield DV-II +Pro UK) at 25±0.5°C. The speed was kept 100 rpm using 6 spindle sizes for 1min.

pH

pH of all five formulation was measured using pH meter (HI 2210 Hanna, USA).

Conductivity

Conductivities of all ME was measured using conductivity meter (Eco Scan, con5, Eutech instrument).

Spreadability

To check the rheological flow ability and spread ability a known weighed cellulose acetate filter paper was taken (W_1) and placed in the center of aluminum foil sheet (Aggarwal *et al*., 2013). The developed ME samples were taken and filled in 5ml syringe. Then fixed numbers of drops (approx. 20) were injected out from the syringe in the center of filter paper. After 10min filter paper gets saturated with formulation was removed away from the unsaturated portion. The unsaturated portion weighed out accurately as (W_2). Percent spread weight was calculated by the following equation.

Spread by weight = $[(w_1-w_2) / w_1] \times 100$…… (i)

Drug content determination

Withania coagulans λ_{max} was determined by preparing stock solution of extract by dissolving 1g in 100ml methanol. Mixture was mixed by vortex and then sonicated for 2mins by ultrasonicator. After that absorbance was taken at respective λ_{max}. Readings were taken in triplicate (n=3).

Particle size determination

Average Droplet size, polydispersity index, and zeta potential of *Withania coagulans* loaded micro emulsion was measured using photon correlation spectrophotometer (Malvern Zeta sizer). The ME sample was placed in cuvette in thermostatic chamber. Sample was diluted with distilled water and readings were taken. Results are shown in table 2.

Screening of different solvent for dilution of extract

Different solvents was used for dilution of extract in order to check the antimicrobial activity. Different solvents used were Dimethyl form amide (DMSO), Tween 80, Ethyl acetate, Petroleum ether, Methanol, Ethanol, and Acetone. That solvent which shows maximum antimicrobial properties against *Withania cagulans* was selected.

Table 1: Composition of extract loaded micro emulsion.

S. no	Frankincense oil	Smix40 (1:1)	Water	Extract
F1	10%	40%	50%	0.8%
F2	15%	45%	40%	1.2%
F3	20%	45%	35%	2.4%
F4	20%	40%	40%	4.8%
F5	15%	50%	35%	10%

Table 2: pH, viscosity, conductivity, spread ability and drug contents of *Withania coagulans* extract loaded micro emulsion. Mean± STD, N=3.

Formulations	pH	Viscosity cP	Conductivity µS/cm	Spread ability %	Extract Content %
F1	5.0 ±0.1	159±0.577	215.1±0.00	95.71±0.115	91.2±0.2
F2	5.2±0.05	446.0±1	189±0.055	95.4±0.1	90.6±0.1
F3	5.08±0.7	981±0.577	176±0.00	95.5±0.1	89.3±0.2
F4	4.4±0.1	393.0±1	185±0.001	93.5±0.057	87.3±0.15
F5	5.48±11	1002±0.72	171±0.001	95.8±0.152	76±0.2

Table 3: Results of globular size, poly dispersity index and zeta potential of *Withania coagulans* extract loaded microemulsion. Mean± STD, N=3

Formulations	Globule size (nm)	Poly dispersity Index	Zeta potential (mv)
F1	127.4±1.61	0.311±0.25	-8±1.0
F2	145±2.56	0.144±0.001	-12±1.0
F3	147.4±1.52	0.288±0.001	-15±1.52
F4	240±2.75	0.477±0.001	-11±1.52
F5	94.38±1.45	0.533±0.002	-14±2.51

Antimicrobial testing of extract

Antimicrobial activity of *Withania Caogulans* extract was evaluated against *Staphylococcus aureus ATCC*#6538, *E. coli* 8739, and *S. typhi* 14028.

Antibacterial activity

For antibacterial activity nutrient ager media was used. 400ml of ager media was prepared and 30ml was transferred to each ager plates for solidification. After solidification microbial culture was added by streaking method. Well diffusion method was used for extract loading. Wells were created by borer and 100µl of *Withania coagulans* fruit extract was loaded in the wells after making dilution of extract in (1:1) from above selected solvents. After that plates were incubated for 24 hrs. After 24hrs zone of inhibition was measured by Vernier caliper against each microorganism. Results are shown in fig. 3 & 4(a).

Antifungal properties of Withania coagulans extract

Withania coagulans extract was evaluated for anti-fungal activity against *Candida albicans* 10231*,* and *Aspergillus niger* 16404.

Saburod ager media was used for antifungal activity against *candida albicans* and *Aspergillus niger*. 30 ml of fungal suspension of *candida albicans* in phosphate buffer solution was prepared and then this was spread over solidified saburod ager media and then wells were created in the SDS media and about 100µl extract dissolved in different solvents as mentioned previously was incorporated in each well. It was than incubated for 48 hrs. After 48 hrs. Zone of inhibition was measured against each microorganism. Results are shown in fig. 4.

Anti-inflammatory activity

Anti-inflammatory activity of *Withania coagulans* extract and extract based micro emulsion was checked by protein inhibition method. Different dilutions of extract were prepared by (50,100,150,200,250, 500, 1000 µg/ 4.5 ml) of 6.8 phosphate buffer solution. To these dilutions 0.45 ml of fresh egg albumin was added and similarly negative control was prepared but without extract. Positive control was prepared by using diclofenac sodium instead of extract in similar dilutions along with fresh egg albumin. After that incubate at 37°C for 10-15 min. After incubation heat on water bath at 57°C for 15 minutes. Denaturation of the protein albumin will occur. Take the supernatant and measure absorbance at 660nm and calculate percent denaturation of protein by using given formula. Similar procedure was done for extract loaded microemulsion by taking 200ug of each formulation.

% Denaturation= [(Absorbance of control - Absorbance of sample)/Absorbance of control] x 100…….. (ii)

STATISTICAL ANALYSIS

Means of all extract loaded formulations was calculated by using Microsoft excel version 10. And standard deviation was calculated by using Origin Pro software version 8.6.1.

RESULTS

Full MS analysis by Electron spray ionization (EPI) performed on *Withania coagulans* fruit extract gives different peaks in range 50-2000 *m/z.* Positive mode gives tentative peaks of *withaferin* A at 493 m/z using sodium ions and at 509m/z using Potassium ions. The tentative peak at 184.08 indicates the presence of *Withasomine* shown in fig. 2.

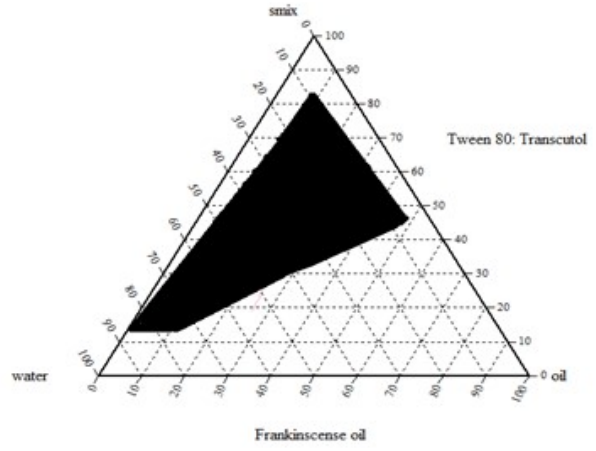


Fig. 1: Pseudo ternary phase diagram using frankincense oil, Smix, and water.

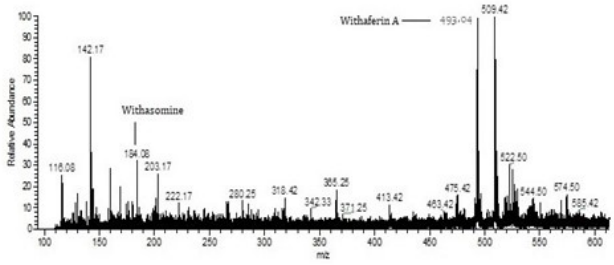


Fig. 2: Relative abundance and *m/z* of *Withania coagulans*

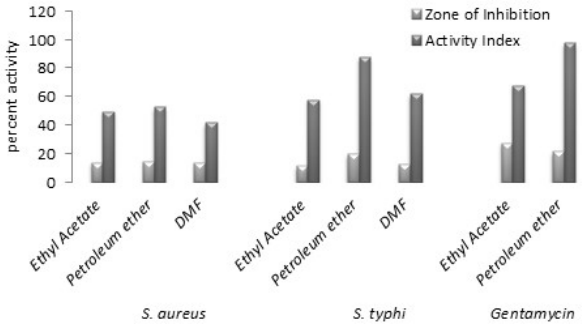


Fig. 3: Zones of inhibition of different microbes using different solvents.

pH, Conductivity and Viscosity

The pH of all Extract based micro emulsion was within range 4 to 5.4. The viscosity was in order F5>F3>F2>F4>F1 i.e. (1002>981>446>393>159cP). Conductivity order of different formulations is F1>F2>F3>F4>F5. Which was within range 85.1±0.00 to.215±0.00 similarly, spread ability was in range 93-95.7%. Drug contents were in range 76-90.6%.

Antibacterial activity

Activity index is calculated by the formula given below Antibacterial activity mentioned in fig. 3 shows that antibacterial activity was 53% against *S. aureus*, 88% against *S. typhi* and (c*) E. coli*. did not show any activity. Gentamycin was taken as control.

Activity index = (Zone of inhibition by test sample/Zone of inhibition by standard) X 100

Antifungal properties

Antifungal activity of *Withania coagulans* checked against *Candida albicans* was found to be 78% and against *Aspergillus niger* was 75% as shown in fig. 4 (a & b).

4(A)

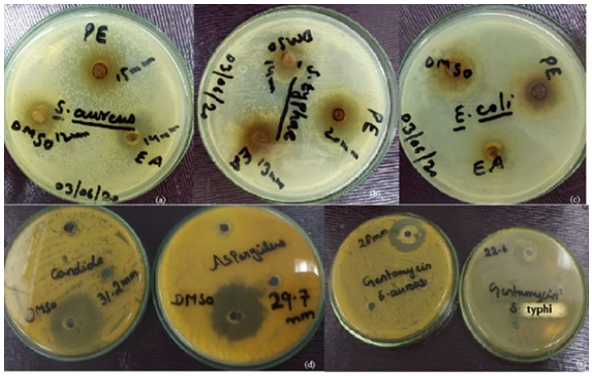
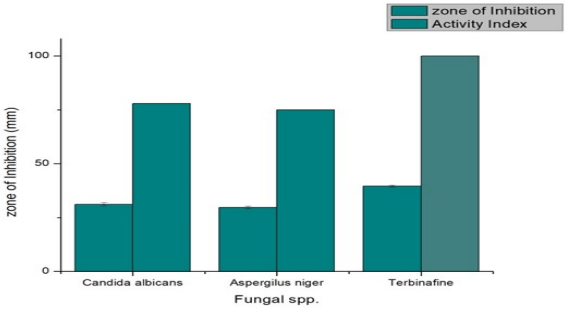


Fig. 4(A): Zone of inhibition by different microbes: (a) *S. aureus*, (b) *S. typhi*, (c*) E. coli*, (d) Antifungal activity by *Aspergillus niger* & *Candida albicans*, (e) Standard drug Gentamycin activity *against S. aureus* & *S. typhi.*

4(B)



4(B): Zone of inhibition and activity index against: *Candida albicans*, *Aspergillus niger* and Terbinafine as standard.

Anti-Inflammatory properties of Withania coagulans

Anti-inflammatory activity of Withania coagulans extract was calculated from the absorbance against different concentration and percentage protein inhibition was found to be maximum 96% and minimum 62%. Anti-inflammatory activity is shown in fig. 5.

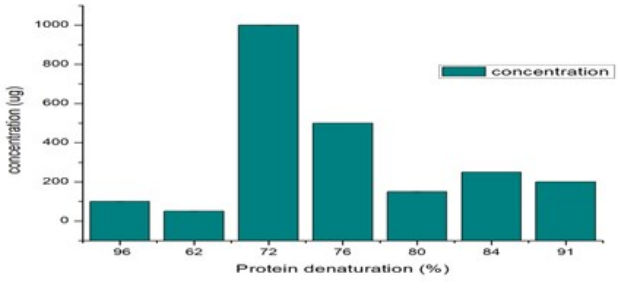


Fig. 5: Anti-inflammatory activity of *Withania coagulans* extract.

Protein denaturation of extract loaded micro emulsion

Protein inhabitation of extract based micro emulsion was calculated in the same manner as of *Withania coagulans* extract by taking 200 µg of all formulations. Order of activity was F5>F4>F1>F2>F3 (90%>89%> 80%>48%> 40%). Maximum activity was shown by F5 as shown in fig. 6.

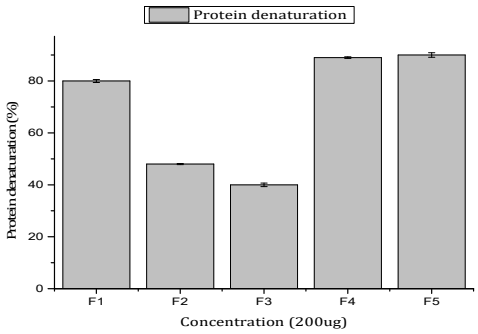


Fig. 6: Graph between and different formulations and % protein denaturation.

DISSCUSSION

The pH of formulation for topical applications ranges 4 to 7. The investigated formulation shows pH ranged from 4.4±0.1 to 5.4±0.11. This is within acceptable limits. The decrease in pH is associated with the increase in water contents of the formulation. Same is the case with our formulations as the percentage of water decrease from 50 to 35% pH decreases. Similar results were reported by (Akram *et al*., 2019).

Conductivity depends on the external phase. Conductivity order of our formulations was F1>F2>F3>F4>F5. Which is within limits 171.5±0.00 to 215±0.00 µs/cm. In case our formulations rate of conductivity increases from 30-50% w/w with the increase in polar phase. Conductivity increases with the increase in water content as found in our investigated micro emulsion. Similar results were reported by (Cojocaru *et al*., 2014) showing decrease in polar phase decreases conductivity.

It has been shown that viscosity of our microemulsion increases with the increase in the concentration of oil (10-20%) and Tween 80: Transcutol ratio from 40-50%. Tween 80 has hydrophilic nature and its structure has polyoxyethylene groups. These groups absorbs aqueous phase of microemulsion resulting in increase in viscosity by reducing free water of formulation. Increase in water contents causes swelling of the microemulsion droplets leading to stronger interaction between interfacial membranes and thus increase in viscosity (Sae Yoon and Sakdiset, 2020). Increase in water contents from 35-50% decreases the viscosity. This is due to the change in the continuity of structure and thus viscosity (Ma and Zhong, 2015). Similar results were reported by (Akram *et al*., 2019). All micro emulsions were easy to spread on skin.

Extract loaded in all formulation was 0.2-10% of the total formulation and extract content of all ME ranges from 76±0.2 to 91.2±0.2%. The results from obtained data indicate uniform distribution of extract.

Globule size is an important measurement to evaluate microemulsion stability, skin permeability and *in vivo* efficacy. Polydispersity indicates uniformity in the formulation. It was noted that globule size is affected by increase in oil/ lipid phase of our micro emulsion as it leads to increase in viscosity and particle size (Dave *et al*., 2017). It was reported in the previous studies that increase in surfactant/co surfactant mixture concentration leads to decrease in the mean droplet size (Prajapati *et al*., 2013). Similar was case with our micro emulsions increase in Smix ratio from 40-50% leads to decrease in droplet size. This is because addition of Transcutol P into the cavities between the Tween 80 causes the interfacial film to condense and stabilize the emulsion, resulting in smaller droplet size (94nm) with polydispersity (0.533).

Polydispersity index is the indication of how particles are uniformly distributed. Polydispersity >0.5 are acceptable because they represent monodisperse system (Ferreira *et al*., 2020). All formulation has polydispersity within range i.e. 0.1 to 0.5 indicating the monodisperse system.

Zeta potential is another factor that counts for stability. Zeta potential between+30 to -30 is acceptable and indication for stability (Kale and Deore, 2017). Zeta potential from F1 to F5 was -8 to -15. This was within acceptable range.

Among DMSO, Petroleum ether and ethyl acetate, maximum zone of Inhibition observed was in order Petroleum ether>Ethyl acetate>DMSO and Zone of

inhibition order was against *S. typhi* > *Staphylococcus aureus* >*E. coli* with diameter of zone of inhibition 20mm > 14mm > 12mm. Activity index order was 88% > 53% > 0%. Zone formation by *E. coli* was not so much clear. Similar studies were reported by (Qasim *et al*., 2020).

Antifungal activity of *Withania coagulans was* in order *candida albicans>Aspergillus niger.* Zone of inhibition was 31.2> 29.7mm. Whereas activity index was found to be 78% and 75% with DMSO showing good antifungal activity of *Withania coagulans*. Studies reported by (Hasan *et al*., 2020) also confirms the antifungal activity *of Withania coagulans*.

The anti-inflammatory activity was found to be higher in case of extract (96%) and extract loaded micro emulsion (90%) in comparison to diclofenac sodium. *Withania coagulans* extract loaded micro emulsions (F5) shows maximum anti-inflammatory activity proving that it has more potent anti-inflammatory activity than diclofenac sodium. Increasing the percentage of extract may further enhance the activity.

CONCLUSION

In this study five formulations were prepared using *Withania coagulans* extract with different percentages. Among them F5 containing Frankincense oil (15%), Smix (50%) and water 35% show good stability, small globule size, high viscosity and maximum anti-inflammatory activity**.** The results suggest there is significant effect of surfactant/co surfactant and water on the droplet size, conductivity and viscosity of micro emulsion. Results also indicate that *Withania coagulans* fruit extract possess very good antibacterial, anti-fungal activity and strong anti-inflammatory activity. Therefore it was concluded that *Withania coagulans* loaded micro emulsion can be used for topical application for treatment of inflammatory skin conditions, alternative to conventional products with fewer side effects. From the In vitro results it is concluded that *Withania coagulans* loaded microemulsions can be successfully used for further *In vivo* studies against inflammatory skin conditions.

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