Development, characterization and evaluation of *in-vitro* anti-inflammatory activity of ginger extract based micro emulsion

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Abstract: *Zingeber officinale* is a commonly used plant which has been shown to possess anti-inflammatory activity. The active compounds present in ginger are gingerols, shagaols and paradol. The aim of this study was formulation of topical microemulsion system to enhance the solubility and stability of ginger extract, as it is unstable in the presence of light, air, heat and long term storage, and to evaluate its anti-inflammatory activity. The solubility of ginger extract in different oils, surfactants, and co-surfactants was determined in order to find the optimal components for microemulsion. IPM was selected as oil phase, tween 80 and PEG 400 were selected as surfactant and co-surfactant respectively based on highest solubility values. Pseudo-ternary phase diagram was constructed in order to find out the microemulsion region. The prepared microemulsions were evaluated for pH, viscosity, conductivity, refractive index, globular size, zeta potential, polydispersity index, ginger extract content. The formulation F1 showed best physicochemical properties with smallest globular size. It also showed significant ($p<0.05$) anti-inflammatory effect as compared to reference piroxicam drug solution. Based on the results, it is concluded that ginger extract can be used to develop stable microemulsion system and promising anti-inflammatory activity.

Keywords: Anti-inflammatory activity, ginger extract, O/W micro emulsion, Pseudo-ternary phase diagram, surfactant.

INTRODUCTION

Zingeber officinale (Ginger) is a herbaceous plant which belongs to family Zingeberaceae and is widely used as herb, condiment and spice (Chan and Wong, 2015). It has been used as traditional medicine from ancient times for the management of several diseases including inflammatory diseases. The active compounds present in ginger are gingerols, shagaols and paradol that have anti-inflammatory, anti-oxidant, anti-cancer and anti-atherosclerotic properties (Habib *et al*., 2008).

Microemulsions (ME) are thermodynamically stable isotropic systems in which two immiscible liquids are mixed to make single phase by the addition of surfactant. They have been used for delivery of both water soluble and lipid soluble moieties. Microemulsions can be used for the delivery of many kinds of drugs and plant extracts because it is thermodynamically stable, easy to prepare, have good appearance, have large surface area with very small particle size (Suresh *et al.*, 2013).

This solubility issue is the major barrier in the drug delivery of new drug and many existing drugs. Microemulsions appear to be the best vehicles for the delivery of these poorly soluble entities. The small droplet size of microemulsions has the advantage of adhering to biological membranes and to transport these entities in more controlled manner. Using ME as drug delivery vehicles, lipophilic components from different plant extracts can be co-solubilized to obtain synergistic effect for various therapeutic purposes Microemulsions has the ability to enhance the solubility, stability and permeability of drugs (Kogan and Garti, 2006). Topical inflammation may occur as a result of various skin diseases and topical NSAIDs like piroxicam, diclofenac, naproxen has widely been used for the treatment of inflammation. But topical NSAIDs have various limitations like skin irritation after application, difficulty in formulation development etc. (McPherson and Cimino, 2013)**.**

The aim of the study is formulation of novel topical microemulsion delivery system of ginger plant because of its potent anti-inflammatory activity and to improve its stability. A study reported that, when gingerol was applied topically, it has been shown to decrease phorbol ester induced inflammation in mice. The basic mechanism of action involves inhibition of COX-1 and COX-2. (Tjendraputra, 2001).

MATERIALS AND METHODS

Ginger rhizome was purchased from the local market, Faisalabad, Pakistan and authenticated by Dr. Adnan

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Amin, Department of Pharmacognosy, GU. The herbarium reference is 45/Phg-GU/2018. Tween-60, Tween-80, Span-20, Span-80, Polyethylene glycol 400, Propylene glycol, Ethyl acetate, Mineral oil and Isopropyl myristate were obtained from Daejung chemicals (Korea). Cremophore EL and Miglyol 812N were purchased from Pakistan scientific store, Faisalabad (Pakistan). Coconut oil, Soybean oil, Almond oil, Eucalyptus oil, Castor oil and Olive oil were purchased from Al-Barkat Pharmaceuticals Ltd. (Lahore, Pakistan). Potassium dihydrogen phosphate (KH_2PO_4) was acquired from E. Merck (Germany). Sodium hydroxide (NaOH) was obtained from Riedel-de Haen (Germany).

Preparation of Ginger Extract

Ginger rhizomes (5kg) were obtained from a local market, Faisalabad, Pakistan. Ginger was washed and cut into small pieces, and then shade dried. Unpeeled ginger was used for solvent extraction because peeled ginger may lose its important components (Nile and Park, 2015). The dried ginger was then milled in a blender to obtain the fibrous powder of ginger. Maceration method was employed to obtain ginger extract. The obtained powder (500g) was macerated with 2.5L of ethanol. After 2 days of maceration the solid plant material was separated from the extract by using filter paper (Whatman No. 1). Rotary evaporator (Stuart, UK) was used to remove or evaporate the solvent from the extract at 45^{o}C. A semi-solid, light brown extract was obtained which was weighed, covered with aluminium foil and stored at 4^{o}C.

Screening of oils, surfactants and co-surfactants

The solubility of ginger extract in various oils (Isopropyl Myristate, Miglyol 812N, Soybean oil, Eucalyptus oil, Coconut oil, Almond oil, Castor oil, Olive oil, and Mineral oil), surfactants (Tween 80, Tween 60, Span 80 and Cremophore EL), and co-surfactants (PG, PEG 400, Span 20 and Ethyl acetate) was determined (Zhang *et al*., 2008) The experiments were run in triplicate.

Pseudo ternary phase diagram construction

The method was adopted from (Alam *et al*., 2009) with slight modifications. Pseudo ternary phase diagram was constructed to find the ME region. Phase diagram was constructed using 1:1 weight ratio of S/Co. Oil and S/Co ratios were mixed carefully at various weight ratios of 0.5: 9.5, 1.0: 9.0, 1.5; 8.5, 2.0: 8.0, 2.5: 8.5, 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 and 9.5: 0.5 (w/w) in 15 different glass tubes. Tubes containing the mixture were homogenously mixed for 2 min by vortex mixer (PCSIR, Pakistan) at ambient temperature. Water was added in small increments of 50 µl. After each increment mixtures were vortexed for 2 min to completely homogenize the sample and next increment was added after every 20 min of vortexing. After homogenization, samples were visually observed against dark background by enlightening the sample with white light to confirm the isotropic nature of sample. Resultant mixtures were named as being transparent, translucent, turbid, gel, or milky in appearance. The phase diagram was constructed by Chemix School 3.60 software.

Preparation of ginger extract loaded microemulsion

From pseudo ternary phase diagram, five formulations were selected on the basis of region obtained from phase diagram. Ginger extract was dissolved in Tween 80 and PEG 400 (1:1) mixture. IPM as oil phase was added to the mixture. Then water was added and the final mixture was vortexed until a clear solution was obtained.

Characterization of microemulsions

The average droplet size, polydispersity index and zeta potential of microemulsions were measured in triplicate using photon correlation spectrometer (Malvern Zetasizer, UK). The ME sample was placed in a cuvette in a thermostatic chamber. Before taking the readings, the ME formulations were diluted with distilled water. The viscosities of formulations were determined at 25 ± 0.5^{o}C using rotational viscometer at 50 rpm with help of spindle 64 for 1 min (Brookfield DV-II+ Pro UK). Conductivities, pH and refractive indexes were also determined using Conductivity meter (EcoScan con5, Eutech Instruments), pH meter (HI 2210 Hanna, USA) and Abbe Refractometer (PCE instruments UK) respectively. Experiments were performed in triplicate for each sample.

Drug content determination

For drug content analysis of ginger extract loaded ME formulations, 1g of sample was taken and diluted with methanol. Then mixture was vortexed thoroughly and sonicated in ultrasonicator machine. Then measurements were taken by UV spectrophotometer by appropriate dilutions with methanol at respective λ_{max}. The measurements were taken in triplicate (n=3).

Estimation of In-vitro anti-inflammatory activity

The anti-inflammatory activity of ideal ginger extract loaded ME formulation was evaluated by protein denaturation study. The procedure was adopted from (Chandra *et al*., 2012) with slight modifications. Different concentrations of 31.25, 62.5, 125, 250, 500, 1000 mg/mL were prepared. 5mL of reaction mixtures were prepared each containing 0.2mL of egg albumin (from fresh hen's egg), 2mL of sample with different concentrations and 2.8mL of phosphate buffer (0.2 M, pH 7.4). Similar volume of egg albumin, phosphate buffer and distilled water was taken as control. The mixtures were placed in incubator (EHRET, Germany) at 37°C for 15min. Then reaction mixtures were placed in water bath at 60°C for 10 min to induce the denaturation. Then samples were cooled and absorbance was measured at 660nm. Piroxicam drug solution with similar concentrations was used as reference drug. Similar procedure was adopted for

reference drug reaction mixtures and absorbance was noted. The %age inhibition of protein denaturation was calculated as follows:

$$\text{Percentage inhibition} = \frac{\text{Control} - \text{Treated Sample}}{\text{Control}} \times 100$$

Stability studies

Stability of microemulsion formulations containing ginger extract was evaluated. 5ml of each formulation was added into glass vials and then sealed and stored at room temperature (25 ± 0.5ºC) for 3 months. Then the samples were analyzed visually for clarity, phase separation and drug precipitation, ginger content, viscosity, conductivity and pH values. For centrifugal stability studies the samples were centrifuged in a centrifuge machine (HERMLE, Germany) at 6000 rpm for 30 min. All experiments were repeated three times (n=3).

STATISTICAL ANALYSIS

The mean and standard deviation of all the results were calculated. All the results among blank and extract loaded ME formulations were treated statistically using one-way analysis of variance (ANOVA). The results between blank and ginger extract loaded ME formulations were compared using student's t-test. The results were statistically significant if $p < 0.05$ and confidence interval was taken 95%.

RESULTS

Solubility studies

From the solubility data represented in fig. 1, it was found that ginger extract showed maximum solubility in Isopropyl Myristate (IPM) which was used as oil phase, tween-80 which was used as surfactant and PEG 400 which was chosen as co-surfactant in microemulsion preparation.

Pseudo-ternary phase diagram

Microemulsion region was identified using pseudo-ternary phase diagram. Phase diagram of IPM, Tween 80 and PEG 400 is presented in fig. 2.

Viscosity

The mean viscosities of blank ME formulations ranged from 24 - 493.93 cP and extract loaded ME formulations showed viscosity range from 26-505.27 cP as shown in table 1.

The results of viscosity among the blank and ginger extract loaded ME formulations were considered significantly different ($p<0.05$) from F1 to F5. Loading of ginger extract cause slight increment in the viscosity of the ME formulations but the viscosity values between blank and extract loaded ME formulations were found to be not different significantly ($p>0.05$).

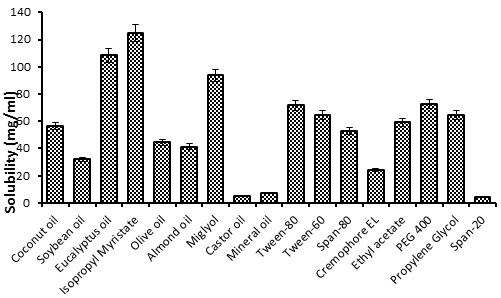

Fig. 1: Solubility data of Ginger extract in various excipients

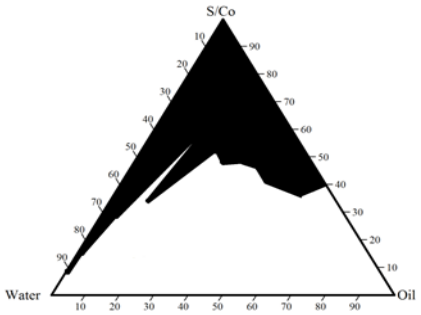

Fig. 2: Pseudo-ternary phase diagram composed of IPM as oil phase, Tween 80 / PEG 400 as Surfactant / Co-surfactant mixture (1:1)

Conductivity

The results of conductivity are shown in table 1. The conductivity values are decreased as we move from F1 to F5. Ginger extract loaded ME formulations and blank formulations show conductivity values to be significantly higher from F1 to F5. But there was no difference significantly in the conductivity values between blank & extract containing ME formulations.

pH measurements

pH values of all blank and extract loaded ME formulations are within the acceptable range (table 1). The pH values were higher significantly ($p<0.05$) among ME formulations with and without extract from F1 to F5. The results between blank ME formulations and formulations containing ginger extract were not significantly different ($p>0.05$).

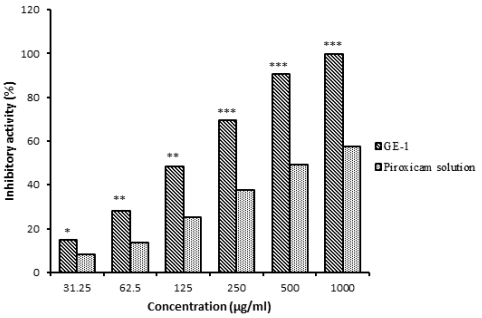

Fig. 3: Inhibition of protein denaturation by Ginger extract loaded ME formulation 1 and Piroxicam solution. (*) represents significant difference of ME formulations in comparison to piroxicam drug solution.

Table 1: Results of apparent viscosity, pH, conductivity and refractive index of blank and ginger extract loaded microemulsion formulations. Mean ± S.D., N=3.

Formulation	Viscosity (cP)		pH		Conductivity(µS/cm)		Refractive Index	
	Blank	Ginger extract loaded	Blank	Ginger extract loaded	Blank	Ginger extract loaded	Blank	Ginger extract loaded
F1	24 ± 12	26 ± 13.11	5.83 ± 0.06	5.93 ± 0.06	195.33 ± 22.55	207.2 ± 18.73	1.3830 ± 0.006	1.3967 ± 0.003
F2	58.67 ± 12.22	60.67 ± 12.06	6.03 ± 0.15	6.13 ± 0.12	132.77 ± 12.35	139.33 ± 13.65	1.3970 ± 0.001	1.4010 ± 0.006
F3	125.33 ± 20.53	134 ± 15.1	6.17 ± 0.06	6.23 ± 0.058	96.63 ± 14.59	105 ± 17.52	1.4054 ± 0.002	1.4145 ± 0.002
F4	316.33 ± 16.26	327.83 ± 15.4	6.33 ± 0.06	6.33 ± 0.058	61.1 ± 10.66	69 ± 12.49	1.4170 ± 0.002	1.4264 ± 0.002
F5	493.93 ± 15.15	505.26 ± 14.1	6.5 ± 0.1	6.57 ± 0.058	43.47 ± 12.04	50.67 ± 14.19	1.4289 ± 0.004	1.4369 ± 003

Table 2: Results of globular size, PDI, and zeta potential of blank and ginger extract loaded microemulsion formulations. Mean ± S.D., N=3.

Formulation	Globular size (nm)		PDI		Zeta potential (mV)	
	Blank	Ginger extract loaded	Blank	Ginger extract loaded	Blank	Ginger extract loaded
F1	21.6 ± 2.39	22.33 ± 2.48	0.148 ± 0.004	0.157 ± 0.007	-22.67 ± 1.15	-22.77 ± 1.88
F2	34.97 ± 3.44	42.05 ± 3.95	0.246 ± 0.006	0.253 ± 0.006	-23.93 ± 0.76	-24.57 ± 0.21
F3	42.71 ± 3.30	48.13 ± 3.37	0.289 ± 0.004	0.287 ± 0.009	-24.43 ± 0.31	-24.83 ± 0.06
F4	61.20 ± 5.89	69.53 ± 6.82	0.305 ± 0.003	0.313 ± 0.006	-26.17 ± 0.35	-26.3 ± 0.36
F5	103.5 ± 4.25	112.4 ± 9.14	0.41 ± 0.006	0.421 ± 0.005	-28.7 ± 0.26	-29.1 ± 0.4

Refractive index

From table 1 it is shown that Refractive index increases from F1 to F5. The R.I values were higher significantly ($p < 0.05$) among ME formulations with and without extract from F1 to F5. The results between blank ME formulations and formulations containing ginger extract were not significantly different ($p>0.05$).

Drug content

Ginger content of all ME formulations containing ginger extract ranged from 98.6±0.74 to 99.6±0.79%. The obtained data revealed the uniform distribution of the drug within all studied ME formulations. The ME formulations were not different significantly ($p>0.05$).

Globular size and Polydispersity index

The results of globular size and PDI are given in table 2. The mean droplet size of blank ME formulations ranges from 21.6-103.5 nm while the mean droplet size of ginger extract loaded ME formulations ranges from 22.33-112.4 nm. Similarly PDI values were from 0 to 1 in range. The Globular size and PDI values were higher significantly ($p<0.05$) among ME formulations with and without extract from F1 to F5. The results between blank ME formulations and formulations containing ginger extract were not significantly different ($p>0.05$).

Zeta Potential measurements

The zeta potential values were higher significantly ($p< 0.05$) among ME formulations with and without extract from F1 to F5 shown in table 2. This is because of increased surfactant and co-surfactant ratios as we move from F1 to F5. The results between blank ME formulations and formulations containing ginger extract were not significantly different ($p>0.05$).

In-vitro anti-inflammatory study

GE-1 formulation containing ginger extract was chosen for *in-vitro* anti-inflammatory study. The %age inhibition of protein denaturation was calculated and compared with control. There was significant difference between percent inhibitions of microemulsion containing ginger extract with different concentrations as compared to control. Similarly the percentage inhibition of protein denaturation by piroxicam solution as standard drug was also calculated and compared with control which showed significant difference. The ME formulation with ginger extract showed significantly higher inhibition ($p<0.05$) of protein denaturation as compared to piroxicam solution as shown graphically in fig. 3.

Stability studies

All ME formulation loaded with ginger extract were evaluated for visual appearance, pH values, viscosity, conductivity and % ginger content at 0 day, 30 days, 60 days and 90 days which showed almost similar results before and after storage at room temperature. The results of centrifugation test indicated no precipitation. ME formulations before and after storage were not different significantly ($p>0.05$)

DISCUSSION

Solubility studies

Based on the solubility studies of ginger extract, IPM oil, Tween 80 as surfactant and PEG 400 as cosurfactant were used as most appropriate combination for micro emulsion. Tween 80 and PEG 400 both are non-ionic in nature and were used at 1:1 ratio for ME formulations preparation.

Pseudo-ternary phase diagram

Microemulsion region was determined from phase diagram. From this region, five different compositions of oil, surfactant and co-surfactant were selected to make five ME formulations. Oil ratios used in all five formulations were 4%, 7%, 10%, 15% and 20 % w/w respectively. Tween-80/PEG 400 was used in ratios of 40%, 45%, 50%, 55% and 55% w/w respectively. Similarly water ratio was 56%, 48%, 40%, 30% and 25% w/w respectively. All formulations were found to be transparent. After incorporation of ginger extract, the color of ME formulations turned to light brown.

Viscosity

It has been shown that there is a significant increase in viscosity as the amount of IPM increased from 4–20%, Smix of tween 80 and PEG 400 increased from 40-45% and water quantity decreased from 25-56% from formulations F1 to F5. This shows that the viscosity increment in formulations was due to increment in the internal phase ratio in o/w ME. As the water content decreases the viscosity of formulation, it is stated in some studies that, the viscosity of ME may be due to oil phase properties and droplet diameter of internal phase. Tween 80 has hydrophilic nature (HLB=15) and its structure has large number of polyoxyethylene groups which tend to absorb the aqueous phase. This results in increase of viscosity due to reduction of free water of the formulations (Mortazavi and Pishrochi, 2013).

Conductivity

Electrical conductivity was measured to examine the type of microemulsion system. The electrical conductivity is different among o/w, w/o and bicontinous ME formulations. This conductivity property is due to presence of external water phase in o/w type. It was reported in a study w/o ME conductivity value is below 10 µS/cm and o/w ME has conductivity range from 10 to above 100 µS/cm (Park *et al*., 2005). The conductivity study of ME showed, with the increase in aqueous phase there is increase in conductivity values (Khalil *et al*., 2012). The decrease in conductivity values as we move from F1 to F5 is due to decrease in water content.

pH measurements

The pH value of the skin ranges from 4.0 – 7.0. As the water content increases from F5 to F1 the pH value decreases. This may be due to low pH value of distilled water used ($pH_{used\ water}$ =5.45) (Cojocaru *et al*., 2014).

Refractive index

R.I is a physical property and is mostly used to confirm the substance nature, to find its concentration and purity. The R.I indicates the isotropy of the microemulsions. As water is the external phase in o/w ME, the R.I value is lower as compared to w/o ME because of lower R.I of water (1.3325). As water content was increased from 25 to 56% from F5 to F1, the refractive index decrease. This is because of lower R.I value of water compared with that of other components of the formulations, i.e. oil or Smix (Olariu *et al*., 2014). Refractive index increases from F1 to F5 as the oil phase and surfactant increases because of large refractive index values of IPM (1.434) and tween 80 (1.473).

Globular size and Polydispersity index

Globular size measurement is an essential parameter of ME as it affects the stability of microemulsion, skin permeation and hence sin-vivo efficacy (Kumari and Kesavan, 2017). The droplet size of ME generally ranges from 100-150 nm. It has been observed that mean particle size decreases with less concentration of oil phase and increased with increase in oil content (Mortazavi and Pishrochi, 2013). It has been reported that, at high S/Co ratios, the long lipophilic chain of tween 80 might cause to increase the droplet size of ME (Kajbafvala *et al*., 2016). The globular size increases as the curvature of surfactant layer becomes more positive. The interfacial activity of Tween 80 has great influence on droplet size of microemulsion (Puri *et al*., 2016). The F1 formulation (both blank and extract loaded) showed minimum particle size because of less oil and surfactant concentrations. The size of extract loaded formulations was slightly higher than blank ME formulations.

Polydispersity index (PDI) is the measure of globule homogeneity. Its value ranges from 0-1. The PT value close to zero indicates the higher homology between the particles (Pandey *et al*., 2014). From the results shown in table 2, it was concluded that PDI values lies within the range (0-1) and all formulations have homogenous size distribution.

Zeta potential measurements

The zeta potential magnitude gives signal of stability among particles of system. When each globule in the microemulsion have a high positive charge or negative charge, there will be repulsion among them and there will be no chance for the particles to come close to each other to form agglomerates It has been observed that ME have good stability if zeta potential is less than – 30mV. Zeta potential values changes with change in surfactant concentrations. The small values of zeta potential revealed the stability of the systems as the droplet aggregation is not expected to take place. The surfactant (tween 80) acts as steric stabilization (Kumari and Kesavan, 2017).

In vitro anti-inflammatory study

Anti-inflammatory activity of GE-1 formulation was calculated because of low globular size, potential and low viscosity. Microemulsion formulation containing ginger extract showed higher inhibition of protein denaturation as compared to piroxicam solution which proves that ginger extract ME formulation has potent anti-inflammatory activity.

Stability studies

There is no precipitation or separation of phases in any of the microeumlsion formulation. The results indicates that all ME formulations were stable physically and chemically.

CONCLUSION

In this study, five ME formulations were prepared and tested for physicochemical properties. The results indicate that formulation GE1 containing 4% IPM, 40% of S/Co ratio and 56% water showed best physical and chemical properties with small globular size and low viscosity. The results suggest that there is significant effect of nature and concentration of surfactant and oil on the rheology and other properties of ME system. The solubility and stability of ginger extract is significantly improved by incorporating in microemulsion system. Ginger extract when loaded in ME system showed potent anti-inflammatory activity as compared to standard piroxicam solution. So it can be concluded that ME formulation loaded with ginger extract can be used as potent anti-inflammatory agent for topical delivery with fewer side effects.

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