

Microemulsion based on *Pterodon emarginatus* oil and its anti-inflammatory potential

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This article reports the development of a pharmaceutical product containing vegetable actives from a Brazilian medicinal plant. The possibility of forming a microemulsion using *Pterodon emarginatus* (“sucupira”) oil was evaluated and the anti-inflammatory potential of this microemulsion was also examined. A formulation was developed using *P. emarginatus* oil, a mixture of ethoxylated Castor Oil (Ultramone® R-540/propylene glycol 2:1) (surfactant/cosurfactant) and distilled water at a ratio of 10:15:75, respectively. The microemulsion which was selected was then subjected to the preliminary stability test and analyzed in terms of average diameter of droplets, pH, zeta potential, and polydispersity index, on the 1st, 7th, 15th, and 30th days after preparation and stored at different temperatures (5 ± 2 °C, 25 ± 2 °C, and 40 ± 2 °C). The anti-inflammatory *in vivo* activity of both oil and formulation were evaluated, using the experimental model of croton oil-induced ear edema. The preliminary stability test showed that the microemulsion stored at 5 and 25 °C retained its original features throughout the 30-day period. The anti-inflammatory potential of both oil and formulation was shown to be statistically significant (p < 0.001), when compared to the control group, however, the microemulsion proved to be more effective (p < 0.05) than the oil when applied directly to the ear.

Uniterms: *Pterodon emarginatus*/pharmacognosy. *Pterodon emarginatus*/oil/anti-inflammatory activity. Medicinal plants. Microemulsion. Phytomedicine. Pharmaceutical technology.

Descrevemos o desenvolvimento de um produto farmacêutico contendo ativo vegetal baseado em uma planta medicinal brasileira. Foi avaliada a habilidade de formação de uma microemulsão à base do óleo de *Pterodon emarginatus* (sucupira) e seu potencial anti-inflamatório. Uma formulação foi desenvolvida utilizando o óleo de *P. emarginatus*, mistura de óleo de mamona etoxilado (Ultramona® R-540)/propilenoglicol (2:1) (tensoativo/cotensoativo) e água destilada, na proporção de 10:15:75, respectivamente. A microemulsão selecionada foi submetida ao teste preliminar de estabilidade e foi analisada quanto ao diâmetro médio das gotículas, pH, potencial zeta e índice de polidispersão, no 1º, 7º, 15º e 30º dias após o preparo e foram estocadas em diferentes temperaturas (5±2 °C, 25±2 °C e 40±2 °C). Avaliaram-se a atividade anti-inflamatória *in vivo* do óleo de sucupira e da formulação, usando o modelo experimental do edema de orelha induzido pelo óleo de cróton. No teste preliminar de estabilidade observou-se que a formulação estocada a 5 °C e a 25 °C mantiveram suas características originais durante 30 dias. O potencial anti-inflamatório de ambos, óleo de sucupira e formulação foi estatisticamente significativo (p < 0.001), quando comparado ao controle, porém a microemulsão foi mais eficiente (p < 0.05) que o óleo aplicado diretamente nas orelhas dos animais.

Unitermos: *Pterodon emarginatus*/pharmacognosy. *Pterodon emarginatus*/óleo/atividade anti-inflamatória. Plantas medicinais. Microemulsão. Fitoterápico. Tecnologia farmacêutica.

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INTRODUCTION

The technological development of research on pharmaceutical forms containing herbal materials has expanded over recent decades and contributed to innovation in the pharmaceutical sector (Bassani, González, Petrovick, 2005; Calixto, 2005; Rocha *et al.*, 2008; Araújo-Júnior *et al.*, 2013; Beringhs *et al.*, 2013; Couto *et al.*, 2013; Klein *et al.*, 2013; Quintão *et al.*, 2013).

Emulsified systems are characterized by their capacity to deliver drugs, and depending on the size of the globule and the process used, they can be classified as macroemulsions, microemulsions or nanoemulsions (Jafari, He, Bhandari, 2007). Microemulsion systems are defined as dispersions of two immiscible liquid phases, usually oil and water, stabilized by the presence of surfactants and cosurfactants at the oil/water interfaces (Lawrence, Rees, 2000; Oliveira *et al.*, 2004; Frange, Garcia, 2009). An average diameter of droplets in a microemulsion generally ranges from 10-100 nm (100-1000 Å) (Shakeel, Ramadan, 2010; Jadhav, Shetye, Kadam, 2010).

Microemulsion formulations can increase drug transdermal permeation, as has been demonstrated in numerous studies (Kreilgaard, Pedersen, Jaroszewski, 2000; Sintov, Sharipo, 2004; Hua *et al.*, 2004; Sintov, Botner, 2006; Sintov, Brandys-Sitton, 2006; Heuschkel, Goebel, Neubert, 2008; Azeem *et al.*, 2009; Neubert, 2011). Several hypotheses have been put forward for the mechanism of drug permeation enhancement. These include increased drug solubility, which fosters a high concentration gradient towards skin, small droplet size and low viscosity which facilitates permeation, increased drug thermodynamic activity in the microemulsion vehicle, and the disruption of the stratum corneum lipid order by microemulsion components (Kreilgaard, 2002; Zhao *et al.*, 2006; Biruss, Kählig, Valenta, 2007; Gupta, Moulik, 2008; Vicentini *et al.*, 2008; Moniruzzaman, Kamiya, Goto, 2010; Shakeel, Ramadan, 2010).

Pterodon emarginatus Vog. Fabaceae, known as “*sucupira*” is a tree species native to the Brazilian savanna (Lorenzi, 2002), whose fruit has shown anti-inflammatory, antiproliferative, and antinoceptive activities (Carvalho *et al.*, 1999; Euzébio *et al.*, 2009; Spindola *et al.*, 2011; Oliveira *et al.*, 2012), based mainly on its diterpene content (Spindola *et al.*, 2011). Although this fruit, which contains large amounts of oil, has been extensively studied both chemically and pharmacologically, there is no microemulsion product on the pharmaceutical market. Consequently, the aim of this study was to develop a microemulsion formulation containing *Pterodon*

emarginatus oil and evaluate it for its anti-inflammatory potential.

MATERIAL AND METHODS

Material

Sorbitan monooleate (Span[®]80) and PEG-54 Castor Oil (Ultramone[®] R540) were donated by Oxiteno. Polysorbate 80 (Tween[®]80) and propylene glycol (DEG), Ethyl alcohol (VETEC) were purchased from Atual Chemical Reagents Co. Ltda. The *Pterodon emarginatus* Vog. Fabaceae oil was obtained through the cold pressing of fruits collected in the town of São Domingos (Goiás - Brazil) (S13°42'12.9" W46°20'35.7"). The plants were identified by Dr. Jose Realino de Paula at the *Universidade Federal de Goiás*. A voucher specimen was deposited in the Herbarium of the *Universidade Federal de Goiás* (UFG), Brazil, and cataloged under number 41714.

Hydrophilic-Lipophilic Balance (HLB) required by *P. emarginatus* oil

To determine the required HLB for *P. emarginatus* oil the surfactants polysorbate 80 (Tween80[®]) with a HLB = 15.0 and sorbitan monooleate (Span80[®]) with a HLB = 4.3 were used. Table I shows the composition by weight of the components. The emulsions were prepared using the classic emulsification method. After preparation, the samples were transferred to a graduated tube and left to stand at room temperature for 24 hours. The HLB required for the *P. emarginatus* oil corresponds to the value of the sample with the lowest creaming percentage (Pereira, 2008).

Surfactant and cosurfactant system

To obtain the *P. emarginatus* oil microemulsions certain surfactant and cosurfactant systems were evaluated to find stable formulations. According to the HLB of the oil the necessary surfactants were chosen. The compositions of the formulations tested are described in Table II. The formulations were evaluated for macroscopic parameters such as the presence of bluish reflection and translucency, and for microscopic parameters, such as Brownian motion of particles and coalescence (Pereira, 2008).

Pseudo-ternary diagram

To find the existence range of microemulsions, pseudo-ternary phase diagrams were constructed using the water titration method (Chen *et al.*, 2004). For each

TABLE I - Proportion of lipophilic (Tween 80[®]) and hydrophilic (Span 80[®]) surfactants used to obtain the required HLB for *P. emarginatus* oil

Test	HLB	Oil (g)	Tween 80 [®] (g)	Span 80 [®] (g)	Water (g)
1	5	2	0.13	1.87	16
2	6	2	0.32	1.68	16
3	7	2	0.50	1.50	16
4	8	2	0.69	1.31	16
5	9	2	0.88	1.12	16
6	10	2	1.07	0.93	16
7	11	2	1.25	0.75	16
8	12	2	1.44	0.56	16
9	13	2	1.63	0.37	16
10	14	2	1.81	0.19	16
11	15	2	2.00	0.00	16

TABLE II - Determination of concentrations in percentages of the components of the formulations tested to determine the surfactant/cosurfactant system

Formulation	Oil	Water	Surfactant		Cosurfactant	
			Tween 80 [®]	Ultramone R 540 [®]	Ethyl alcohol	Propylene glycol
1	6.5	57	12.5	-	24	-
2	5	80	10	-	-	5
3	10	75	10	-	5	-
4	10	75	-	10	-	5
5	10	75	-	10	5	-

phase diagram a specific surfactant/cosurfactant weight ratio (Ultramone[®] R-540/propylene glycol 2:1) was used. The ratios of *P. emarginatus* oil to the mixture of surfactant/cosurfactant and water were varied every 10% covering the entire area of an equilateral triangle. After being equilibrated, the mixtures were assessed visually and classified as being either microemulsions, emulsions or gels.

Microemulsion preparation

Microemulsions were prepared by reverse-phase methods. The water phase and oil phase with surfactants were heated separately to a temperature of 75±5 °C. The aqueous phase was added into the oil phase under magnetic stirring at room temperature (25 °C).

Characterization and stability of microemulsions

To evaluate microemulsion stability, stress conditions including aging, temperature and centrifugation were applied. The formulations were also subjected

to centrifugation at 3000 rpm for 15 minutes at room temperature and samples were withdrawn after a particular time interval and observed for phase separation. The formulations selected were kept at three different temperatures i.e. 4 °C, 25 °C, 40 °C and observed for phase separation, pH, zeta potential and average particle size on the 1st, 7th, 15th, and 30th days after preparation (ANVISA, 2004; Moghimipour, Salimi, Leis, 2012).

pH

The pH of the formulation was carried out using a Tecnal TEC 3MP pH meter (Tecnal, Piracicaba, São Paulo, Brazil). Analyses were performed in triplicate inserting the electrode directly into the sample without dilution (ANVISA, 2010).

Zeta potential

Zeta potential was determined by measuring the electrophoretic mobility of dispersed particles in an

electric field (ZetaPlus, Brookhaven, New York, USA). The formulations were diluted in distilled water (1:15), homogenized until completely dispersed and then subjected to analysis, which was performed in triplicate (Silveira, 2009; Moghimipour, Salimi, Leis, 2012).

Dynamic light scattering (DLS)

The particle size of the microemulsions and the polydispersity index were determined by the laser light scattering technique (Malvern Zetasizer Nano S, Malvern, UK). The sample was diluted with distilled water (1:10) and placed in cuvettes of 1 cm³ volume at a temperature of 25 °C. The measurements were performed in triplicate (Kale, Allen Jr, 1989; Silveira, 2009; Moghimipour, Salimi, Leis, 2012).

In vivo experiments

Animals

The experiment was conducted with male Swiss mice weighing from 40 to 45 g. They were kept under controlled temperature and light conditions (light/dark cycles of 12 h) with food and water *ad libitum*. The anti-inflammatory studies were carried out in accordance with current norms of the Brazilian College of Animal Experimentation (COBEA), and authorized by the Research Ethics Committee (number 104/08) at the *Universidade Federal de Goias*.

Croton Oil-induced Ear Edema

The ear edema was induced as in the procedure previously described by Tubaro *et al.* (1985) cited in Pinheiro *et al.* (2011) with modifications. Three groups with 10 mice each were used. Inflammation was induced by applying 25 µL of acetone solution containing the irritant croton oil 2.5% v/v to the inner surface of the right ear of each animal. The left ear was used as a control and received 25 µL of vehicle (acetone). Thirty minutes after applying the irritant, the mice were treated with 25 µL of the microemulsion formulation without the oil (negative control), 25 µL of *P. emarginatus* oil in acetone (100 mg/mL), and 25 µL of the microemulsion containing the *P. emarginatus* oil.

Four hours after applying the phlogistic agent the animals were euthanized and 6 mm discs were taken from both ears of each animal. The difference in weight between the discs was taken as the measurement of the edema. The anti-inflammatory activity of oil and formulation was evaluated by the difference between the values obtained for the negative control and those of the other groups.

Statistical Analyses

The results were expressed as mean ± S.D. For the group comparisons, one-way layout analysis of variance was applied. The data were analyzed statistically using variance analysis following Tukey's comparison test. P values less than 0.05 were considered as indicative of significance using GraphPad Prism 4 (GraphPad software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Hydrophilic-Lipophilic Balance (HLB) required by *P. emarginatus* oil

The HLB required for the *P. emarginatus* oil was between 14 and 15 since these values presented a higher percentage of creaming as shown in Table III. The required HLB values were evaluated for the first time. The HLB parameter is an important tool for the technological development of phytopharmaceuticals and can be successfully employed in the formulation of natural products such as those based on vegetable oils (Fernandes *et al.*, 2013)

TABLE III - Creaming profile obtained with the Tween 80® and Span 80® surfactant assessment for the HLB of *P. emarginatus* oil

HLB	% Creaming
5	-
6	3.45
7	6.90
8	13.79
9	6.90
10	13.79
11	65.52
12	51.72
13	75.86
14	79.31
15	82.76

Surfactant and cosurfactant system

The formulation with Ultramone R-540®/Propylene glycol showed better results when the parameters were compared (Table IV). According to Mittal (1999), the possibility of forming microemulsions depends on the balance between the hydrophilic and lipophilic

TABLE IV - Macroscopic and microscopic analysis of formulations tested to evaluate the surfactant system with increased stability

Formulation	Macroscopic and microscopic parameters				
	Bluish reflection	Translucency	Brownian motion	Particle homogeneity	Coalescence
1	-	+	+	-	+
2	+	+	+	-	+
3	-	-	+	-	+
4	+	+	+	+	-
5	+	+	+	-	+

properties of the surfactant, which is determined not only by its chemical structure, but also by other factors such as temperature, ionic strength and the presence of cosurfactant. Pereira (2008) used the same parameters but for olive oil, grape seed oil and a mixture of both and found that the systems showed optimal stability when using a pair of surfactants (Ultron R300® and Span® 80) whose HLB was equal to 8. Kale, Allen Jr (1989), Yagmur *et al.* (2002), Yotsawimonwat *et al.* (2006) and Moghimipour, Salimi, Leis (2012) used propylene glycol as co-surfactant in different formulations of microemulsions.

Phase diagram

Pseudoternary phase diagrams with various weight ratios of surfactant/cosurfactant, *P. emarginatus* oil and water were prepared. The region circled in Figure 1 (shown as number 22) represents the microemulsion formation regions.

Formulation 22 (10% *P. emarginatus* oil, 20% S/Co-S, and 70% water) demonstrated greater stability for phase separation, as well as presenting the typical characteristics of the microemulsion, such as a bluish reflection, translucence, and absence of coalescence when analyzed under an optical microscope. Formulations 11, 12, 13, 16, 17, 18, 23, 24, 29, 30, and 31 formed unstable emulsions when phase separation was complied with after centrifugation. The other formulations did not form emulsions showing gelified aspect with the separation of phases. The experimental results are described in Figure 1.

Based on these results, a new diagram was constructed around the Formulation 22 region to assess system behavior in relation to minor variations in the proportions of the components of the system. The new proportions analyzed are expressed in Table V.

The phase diagram presented in Figure 2 shows seven formulations (37, 38, 39, 40, 41, 45 and 47) with the characteristics of a microemulsion. All formulations presented closely resembling characteristics, but Formulation 40, which had the greatest concentration of

P. emarginatus oil and a lower amount of surfactants in its composition, was selected for further study of its stability.

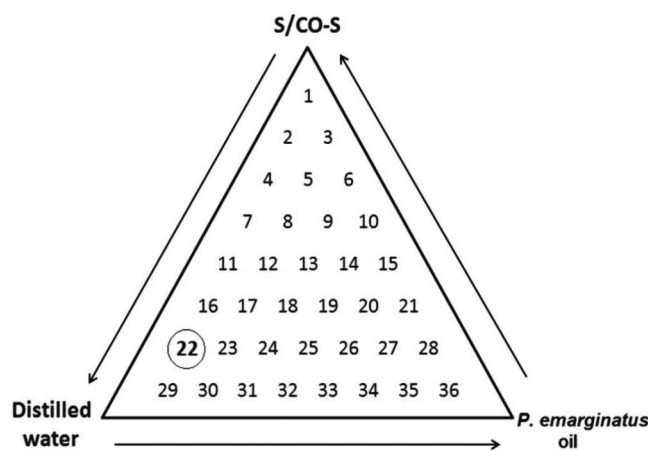


FIGURE 1 - Pseudo-ternary system for water, oil, surfactant/cosurfactant (S/Co-S).

TABLE V – Percentage concentration (w/w) of the components of the formulation for the preparation of a new phase diagram in the microemulsion region

Formulation	Oil	S/Co-S	Water
37	5	20	75
38	5	25	70
39	5	30	65
40	10	15	75
41	10	25	65
42	15	20	65
43	15	10	75
44	15	15	70
45	5	15	80
46	20	15	65
47	5	10	85
48	10	10	80
49	20	10	70
50	25	10	65

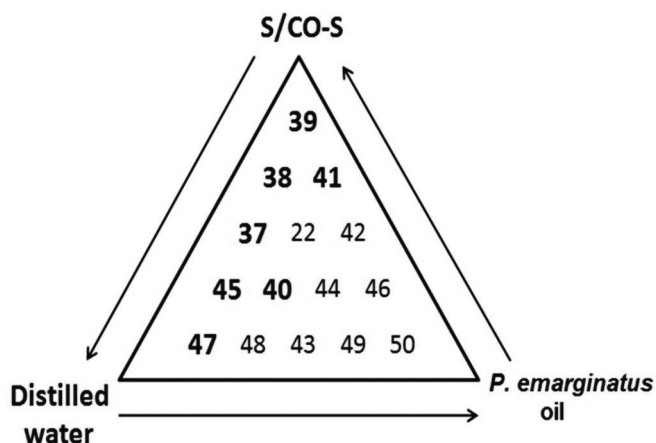


FIGURE 2 - Phase diagram in the surroundings of Formulation 22. Formulations presented in bold have the characteristics of a microemulsion while the other formulations presented only certain features of an emulsion.

The results obtained with the construction of the phase diagram are inherent to the oil and to the surfactant/cosurfactant system used. However, Zhang, Michniak-Kohn (2011) using Labrasol® (Caprylocaproyl macrogol 8 glycerides) and Cremophor EL® (Polyoxyl 35 Castor oil), also noted that the formulations which showed the characteristics of a microemulsion were those in which the proportion of oil was lower than the ratio of surfactant/cosurfactant. Some studies are not consistent with the optimal microemulsion concept of high water content, a balanced quantity of oil and small amount of surfactant. Such studies describe optimal microemulsion formulations with a relatively high mixture of surfactants. In all those cases, the microemulsion systems under investigation used short-chain alcohol, such as ethanol, as the co-surfactant (Yuan *et al.*, 2006; Zhu *et al.*, 2008). Some surfactant molecules are known to self-assemble in polar organic solvents, such as propylene glycol, glycerol, and formamide. These solvents, like water, form hydrogen bonds and have relatively high dielectric constants. Critical micelle concentrations are higher in polar nonaqueous solvents than in water. When these solvents are used as water substitutes, their resulting penetration into the surfactant interface leads to smaller or no liquid crystal phase regions. Alcohols and polyols destabilize the liquid crystalline phase and extend the isotropic region to higher surfactant concentrations (Yaghmur *et al.*, 2002).

Characterization and stability

The microemulsion obtained retained its original features when stored at temperatures of 5 °C and 25 °C.

The formulation maintained at 40 °C showed a significant increase in droplet size and zeta potential and a reduction in pH (Figures 3, 4, and 5). Such variations could be justified by the fact that the physical stability of emulsions is generally intrinsically linked to the temperature to which the product is exposed (Chorilli *et al.*, 2009).

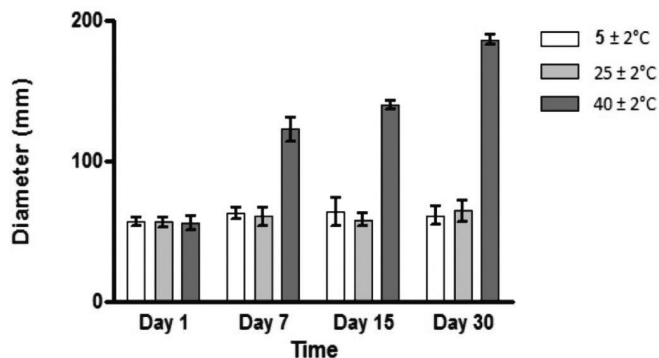


FIGURE 3 - Change in mean diameter of droplets of internal phase as a function of time. Data expressed as mean and standard deviation (n=3).

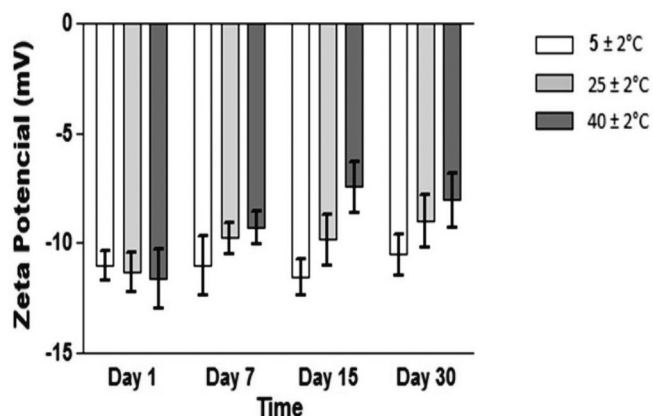


FIGURE 4 - Variation of zeta potential versus time. Data expressed as mean and standard deviation (n=3).

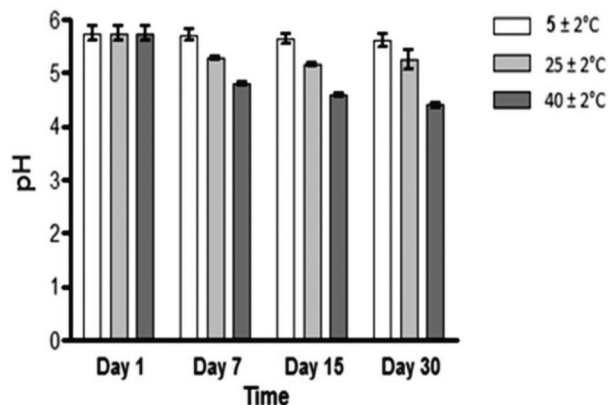


FIGURE 5 - Changes in pH as a function of time. Data expressed as mean and standard deviation (n=3).

The decrease in pH led to an increase in zeta-potential, which can be explained by the mechanism of load generation at the interface. The increase in negative charge density increases the concentration of hydroxyl ions in a solution that tends to favor an increase in protons and a basic pH. On the other hand, a decrease in zeta potential, in module, as well as a more acidic pH can be explained by the neutralization of the negative regions as a result of the adsorption of hydrogen ions (Gallardo *et al.*, 2005; Arias *et al.*, 2008). The polydispersity index showed that all the microemulsions had narrow size distribution (Figure 6).

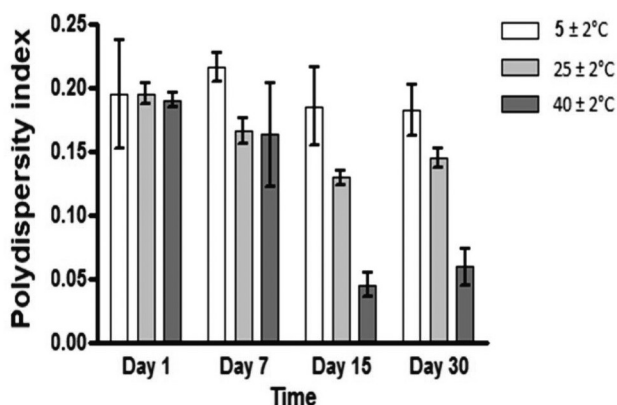


FIGURE 6 - Polydispersity index as a function of time. Data expressed as mean and standard deviation (n=3).

Anti-inflammatory Activity

The results obtained in the evaluation of the anti-inflammatory activity of the microemulsion system (Figure 7) show that both the formulation and the oil were statistically significant when compared to the negative control ($p < 0.001$). The efficacy of the treatment was also shown

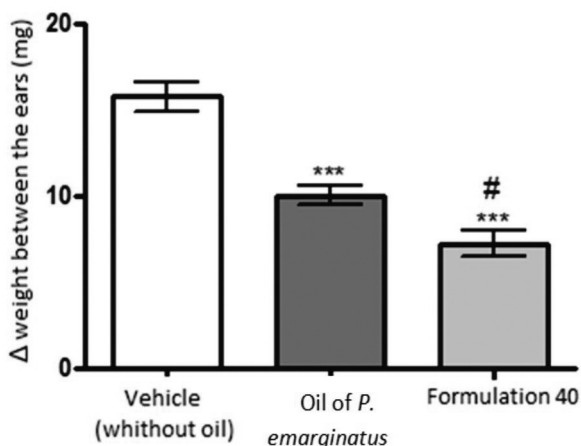


FIGURE 7 - Ear edema, in mg, induced by croton oil (2.5% v/v in acetone) in groups previously treated topically. *** $p < 0.001$ (ANOVA, Tukey test) for treated groups versus control group. # $p < 0.05$ (ANOVA, Tukey test) between treated groups.

when the use of the microemulsion with oil was compared to the application of the oil itself directly to the skin ($p < 0.05$).

After carrying out the experiment we set out to see if the anti-inflammatory potential of the oil was retained after producing the microemulsion. The results obtained for *P. emarginatus* oil are consistent with those studies which show the anti-inflammatory activity of compounds, such as α -humulene and β -caryophyllene and vouacapanes (Spindola *et al.*, 2011). Pinheiro *et al.* (2011), while employing the same experimental model, obtained a 76.63% inhibition of edema using the essential oil *Peperomia serpens*, which is rich in both substances. Fernandes *et al.* (2007) also obtained positive results for different experimental models of inflammation using α -humulene and β -caryophyllene, administered orally, isolated from the essential oil of *Cordia verbenacea*.

The use of microemulsions as a carrier vehicle in the topical formulations of drugs has been shown to improve the permeability of such formulations (Dantas *et al.*, 2010; Hathout, Woodman, Mansour, 2010; Jadhav, Shetye, Kadam, 2010; Rogerio Dora, Andrade, 2010; Tsai *et al.*, 2010). Bolzinger *et al.* (2008) showed that microemulsions based on caffeine have better permeation than pharmaceutical forms, such as emulsion and gel. Problems such as low drug bioavailability and low penetration of formulations used topically in the different skin segments have been cited (Kreilgaard, 2002).

This study presents the first description of developing a potential anti-inflammatory microemulsion using “*sucupira*” oil as a basis. Further studies should be undertaken to investigate the permeation of the formulation on *in vitro* models, evaluate its anti-inflammatory mechanism of action, define pharmaceutical forms to serve as a microemulsion vehicle and perform pre-clinical and clinical trials, in the near future, in order to contribute to innovation in the phytomedicine market.

CONCLUSION

A potential anti-inflammatory phytomedicine using *P. emarginatus* oil as a basis for use in pharmaceutical forms of microemulsion was formulated and characterized. This microemulsion is comprised of *P. emarginatus* oil (10%), a mixture of Ultramone® R-540/ propyleneglycol in a 2:1 ratio (15%) and water (75%). The average droplet diameter of the internal phase was 56.8 ± 6.07 nm, pH was 5.74 ± 0.19 , zeta potential was -11.3 ± 1.54 mV while the polydispersity index showed that the microemulsion had narrow size distribution. Finally, we showed the potential anti-inflammatory activity of “*sucupira*” oil

and its efficacy as a vehicle in the pharmaceutical form of microemulsion.

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