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Physicochemical/photophysical characterization and angiogenic properties of *Curcuma longa* essential oil

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ABSTRACT

This study analyzed the physicochemical and photophysical properties of essential oil of *Curcuma longa* and its angiogenic potential. The results showed that curcumin is the main fluorescent component present in the oil, although the amount is relatively small. The experimental chorioallantoic membrane model was used to evaluate angiogenic activity, showing a significant increase in the vascular network of *Curcuma longa* and positive control groups when compared to the neutral and inhibitor controls ($P < 0.05$), but no significant difference was found between *Curcuma longa* essential oil and the positive control ($P > 0.05$). Histological analysis showed extensive neovascularization, hyperemia and inflammation in the positive control group and *Curcuma longa* when compared to other controls ($P < 0.05$), characteristic factors of the angiogenesis process. In conclusion, *Curcuma longa* oil showed considerable proangiogenic activity and could be a potential compound in medical applications.

Key words: angiogenic activity, curcumin, essential oil, physicochemical.

INTRODUCTION

In recent years the search for materials that stimulate the angiogenesis process has received considerable attention. In particular, biomaterials produced from natural rubber latex have shown biocompatibility as well as stimulating angiogenesis, cell adhesion and extracellular matrix formation (Floriano et al. 2013, Mrue et al. 2004). Angiogenesis is a complex biological process that favors the formation of new blood vessels from preexisting vascular tissue by proliferation, migration, regulation and differentiation of vascular cells (Folkman 2003). From the point of view of medical applications, materials that induce angiogenesis are important for tissue engineering, to enhance cell proliferation or promote wound healing (Schulz et al. 2003, Shen and Falanga 2003).

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Medicinal herbs used in folk medicine have proven to be an important source of compounds with potential for development of a number of medications (Iwu et al. 1994, Phillipson 1994). The various plants used in folk medicine include different turmeric extracts (*Curcuma longa* L.). *C. longa* is a rhizome-bearing perennial herb from the Zingiberaceae family, which is native to Southern Asia and cultivated extensively throughout the warmer climates of the world (Mostajeran et al. 2014). In Brazil, *C. longa*, widely used as a spice, food preservative and coloring, is frequently confused with true saffron, a Mediterranean plant not cultivated in the country (Mata et al. 2004).

There are several data in the literature indicating a wide variety of pharmacological activities of *C. longa*, which exhibits anti-inflammatory, antihuman immunodeficiency virus, antibacterial, and antioxidant effects, as well as nematocidal activities (Araújo and Leon 2001, Jurenka 2009, Nasri et al. 2014). In particular, the essential oil extracted from the rhizome has been shown to possess anti-inflammatory activity, increasing bile flow and efficacy against bronchial asthma (Srimal 1997).

The aim of this study was to evaluate the potential of *C. longa* essential oil as a biomaterial to stimulate angiogenesis. Angiogenic potential was assessed *in vivo* through the chick embryo chorioallantoic membrane model (CAM). In order to obtain physicochemical and photophysical characterization of the essential oil, different techniques were employed, such as: Thermogravimetric analyses (TGA), Fourier transform infrared (FTIR), UV-Vis absorption and fluorescence spectroscopies.

MATERIALS AND METHODS

Extraction of Saffron Essential Oil

Essential oil was extracted from *C. longa* rhizome powder using the hydrodistillation method with steam distillation. In order to avoid significant losses and minimize errors during the process, the operation took place in a closed circuit with the aid of a Clevenger apparatus (Santos et al. 2004). *C. longa* rhizome powder (150 g) was added to 500 ml of distilled water in a round-bottom glass flask. The powder was distilled for 180 minutes and the distillate separated into two phases, an aqueous phase at the bottom of the separator tube and an oil phase at the top. The oil was stored in a sterilized test tube under cooling and light, and identified.

Physicochemical and Photophysical Characterization

FTIR spectra were recorded with a Vertex 70 Bruker spectrophotometer. The FTIR spectra were obtained in the attenuated total reflectance (ATR) mode, in the 400–4000 cm^{-1}range. TGA was carried out with a Shimadzu DTG 60/60H thermoanalyzer. Dynamic scans were conducted at a temperature range between 43 and 600ºC, at constant heating rates of 10ºC min^{-1}, under air atmosphere with a flow of 50 $mLmin^{-1}$ using an alumina crucible. The weight of the material was 13.504 mg. UV-Vis absorption measurements were carried out with a Perkin Elmer Lambda 1050 UV/Vis/NIR spectrophotometer. Excitation, steady-state emission and spectra were recorded by a Horiba Jobin Yvon FluoroMax-3 spectrofluorometer. Time-resolved fluorescence was acquired using an apparatus based on the time correlated single photon counting (TCSPC) method. The excitation source was a titanium–sapphire laser (Tsunami 3950–Spectra Physics), pumped by a second harmonic diode-pumped Nd:YVO_4 laser (Millenia–Spectra Physics), with frequency doubled to 465 nm in an LBO crystal (GWN-23PL–Spectra Physics); additional details can be found in (Rodrigues et al. 2015, Sampaio et al. 2012).

Angiogenic Potential / CAM Assay

The CAM model was used to evaluate angiogenic activity according to a previously described

methodology (Melo- Reis et al. 2010, Ribatti et al. 2000). Sixty fertile chicken eggs (*Gallus domesticus*) of the Ross lineage were incubated at 37°C in a humidified atmosphere (60-70% relative humidity). On the 5th day of incubation, a circular hole was opened in the large end of the eggshell, the CAM membrane removed, and the eggs returned to the incubator. Filter paper disks were soaked in 5 μL of the following solutions: 1. *C. longa* essential oil; 2. Regederm® - a commercial product from Pele Nova Biotecnologia prepared with *Hevea brasiliensis* latex (positive control); 3. water (neutral control); and 4. dexamethasone 4mg/ml (inhibitor). After receiving the treatment solutions, the filters were placed on top of the growing CAM on day 13 of incubation under sterile conditions. The angiogenic response was evaluated 72 hours after incubation. CAMs were fixed in formaldehyde solution (3.7%) for 10 minutes, cut with blunt curved scissors and kept in Petri dishes in the presence of formaldehyde solution. Analysis and quantification of a newly-formed vascular net were conducted through captured images. The area of each assay was determined using *Gimp for Windows* (version 2.0.5) and *Image J* (NIH) imaging programs (version 1.28). The images were prepared so that saturation, light and contrast allowed better resolution of the blood vessels, which were quantified in each corresponding pixel (Parente et al. 2011). In order to analyze the angiogenic activity of *C. longa* essential oil, the treated and control groups were compared using one way analysis of variance (ANOVA) of ranks, followed by Tukey's test (n=10 membranes). P values of less than 0.05 ($P<0.05$) were considered significant.

Histology of the CAM Blood Vessels

The CAM membranes fixed in formaldehyde solution (3.7%) were processed, sealed in paraffin, sectioned with a Spencer micrometer at a thickness of 5 μm, marked by hematoxylin and eosin and examined by microscopy (Zeiss – Axioskop). Slides were examined by an experienced pathologist blinded to the treatment, and photographs were representative of five membranes per group. The following parameters were evaluated: the presence of inflammatory elements, hyperemia and neovascularization. The results were visually classified according to intensity, and the data were transformed into quantitative variables, assigning the following scores: absent (0), slight (1 to 25%), moderate (26 to 50%), and accentuated (over 51%). The results were evaluated using the Kruskal-Wallis test with a significance level of 5% ($P < 0.05$).

RESULTS AND DISCUSSION

Physicochemical and Photophysical Characterization

FTIR spectroscopy, an effective analytical tool for detecting functional groups and characterizing covalent bonding, has been employed to study biological systems and their processes (Gonçalves et al. 2006). Figure 1a shows the FTIR spectra of *C. longa* tumeric powder and essential oil extracted from *C. longa*. The FTIR spectra of *C. longa* obtained here is in agreement with the literature (Shameli et al. 2014). The FTIR results indicate that extraction of the essential oil from the rhizome causes a significant reduction in the strong broad bands between 3100 and 2900 cm^{-1}, which are attributed to bonded hydroxyl (–OH) or amine groups (–NH) and aliphatic C–H of *C. longa* tumeric powder extract. Moreover, a decline was observed in the 1157 and 1020 cm^{-1} bands attributed to C–O (stretching vibrations of oligosaccharides) and C–4–OH (typical of disaccharides), respectively (Anastasaki et al. 2010, Mohanty and Sahoo 2010, Ordoudi et al. 2014, Shameli et al. 2014). This indicates that the extraction process produces the same components as those of the initial material, but there may be a polymerization process that increases oil chains.

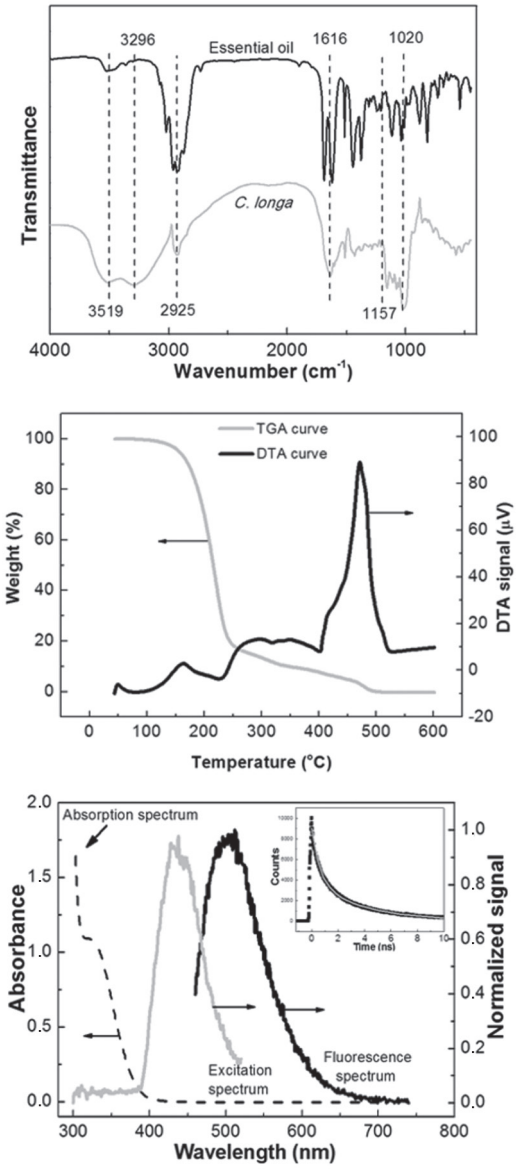

Figure 1 - (**a**) FTIR spectra of *C. longa* tumeric powder and essential oil extracted from *C. longa*, (**b**) TGA and DTA curves of essential oil, and (**c**) UV-Vis absorption, normalized emission and normalized excitation spectra. The inset shows the time-resolved fluorescence of essential oil of *C. longa*.

Thermogravimetric analysis (TGA) makes it possible to study changes in the physical and chemical properties of materials as a function of increasing temperature (Figure 1b). The TGA curve shows high essential oil thermal stability up to 90ºC. In the 90 - 270ºC range there is a significant loss of mass (around 84%), possibly associated with two nearly overlapping endothermic processes shown on the DTA curve. These endothermic peaks are associated with evaporation of low molecular weight organic molecules. A loss of weight of around 9% can be observed in the 270 – 408ºC range, corresponding to a large exothermic band. In the 462 to 500ºC range there is an intense exothermic peak at 472ºC, which may be associated with oxidation/combustion reactions of high molecular weight molecules formed during the extraction process. This result is in line with the enhancement of C – H and C – C vibrations observed in the FTIR results.

C. longa essential oil is a natural yellowish-orange compound soluble in polar organic solvents. Thus, photophysical characterization is a valuable study to identify the chromophore and fluorophore compounds present in the sample. UV-Vis absorption and fluorescence emission spectra for essential oil diluted (20 x) in DMSO are shown in Figure 1c. Intense absorption in the UV region can be observed, with a shoulder close to 322 nm. A broad and structureless fluorescence band can be obtained with excitations between 400 and 480 nm. The fluorescence spectra recorded at different wavelengths exhibit no wavelength dependence. The fluorescence decay curve was obtained using the time-correlated single photon counting method shown in the inset of Figure 1c. The oil was excited at 430 nm and the emission was collected at 505 nm. Time-resolved fluorescence analysis shows a double-exponential fit with lifetimes of 0.5 ns (51%) and 3.3 ns (47%).

Fluorescence excitation spectra indicate that the main fluorescent component absorbs from 400 to 500 nm. Although essential oil of *C. longa* is a complex mixture of chemical compounds, the results obtained by excitation and fluorescence spectra suggest that curcumin is the main fluorescent component (Ghosh et al. 2011, Erez et al. 2014). The fact that the excitation spectra do not match the absorption spectra suggests that

fluorescent resonance energy transfer (FRET) occurs from absorber compounds, absorbing in the 300 - 400 nm range, to fluorescent curcumin, a fluorescent acceptor compound that emits from 425 to 700 nm. The FRET process, a physical phenomenon present in some biological systems, may take place when a donor compound in an electronically excited state transfers its excitation energy to a fluorescent acceptor compound, which, in turn, emits fluorescence (Lakowicz 2006).

The fact that the absorption band of curcumin is not observed in the UV-Vis spectra (Figure 3) leads us to believe that there is only a small amount of curcumin in the essential oil. This corroborates the literature, which generally shows that the major components of essential oil, accounting for around 80% of the total, are ar-turmerone, α-turmerone and β-turmerone, while curcumin represents about 1% (Péret-Almeida et al. 2008, Ferreira et al. 2013).

With respect to the time-resolved fluorescence measurements, the fast component observed may also be attributed to curcumin, which exhibits a fluorescence lifetime of dozens to hundreds of picoseconds (Erez et al. 2014). On the other hand, the long-lasting component could be attributed to residues or other compounds formed during the oil extraction process.

Angiogenic Potential - CAM Assay

Angiogenesis is a process in which new capillaries sprout from preexisting vessels (Chen et al. 2011). Angiogenic drug research, an important area in modern biomedical science, involves primarily the search for plant-based compounds. More than 50% of all drugs in clinical use are of natural origin, which shows the importance of plants as a potential source of therapeutic agents (Majewska and Gendaszewska-Darmach 2011).

Figure 2a shows images of different CAMs obtained in the present study. As can be seen, more and thicker blood vessels were formed in two groups: *C. longa* essential oil and the positive control (Regederm®), when compared with the neutral control (water) and the angiogenic inhibitor (Dexamethasone) groups. Moreover Fig. 2b shows the percentage of vascularization as measured by CAM image analysis. For *C. longa* essential oil the mean vascularization percentage was 46.4 ± 4.6; for the positive control, 53.3 ± 4.3; for the neutral control, 32.5 ± 3.3 and for the angiogenic inhibitor, 12.3 ± 2.0. *C. longa* essential oil showed a significant increase in the vascular network formed, compared to the neutral control ($P < 0.05$) and the inhibitor ($P < 0.05$). No significant difference was observed between *C. longa* essential oil and the positive control.

After CAM image analysis, the membranes were submitted to histological analysis (Figure 3). The results showed an increase in the number of new blood vessels (neovascularization), inflammatory elements and hyperemia for CAMs submitted to *C. longa* latex and Regederm® when compared to the neutral control and inhibitor group ($P < 0.05$). The literature reports that the inflammatory cells are important in activating factors to stimulate the angiogenic process (May et al. 2008). Inflammatory cells such as macrophages, lymphocytes, mast cells and fibroblasts are capable of stimulating vessel growth (Folkman and Brem 1992). Thus, angiogenesis and inflammation complement each other. Our histological data is in accordance with CAM images, which showed significant differences in the presence of inflammatory elements, hyperemia and neovascularization (Table I).

A number of plants and their different extracts have been investigated in relation to their angiogenic potential. Studies have demonstrated significant proangiogenic activity in the following species: *Aloe vera* (Atiba et al. 2011), *Hippophae rhamnoids* L (Gupta et al. 2008), *Angelica sinensis* (Meng et al. 2008), *Cinnamomum cassia* (Choi et al. 2009), *Astragalus membranaceus* (Zheng et al. 2011), *Stewartia koreana* (Lee et al. 2010),

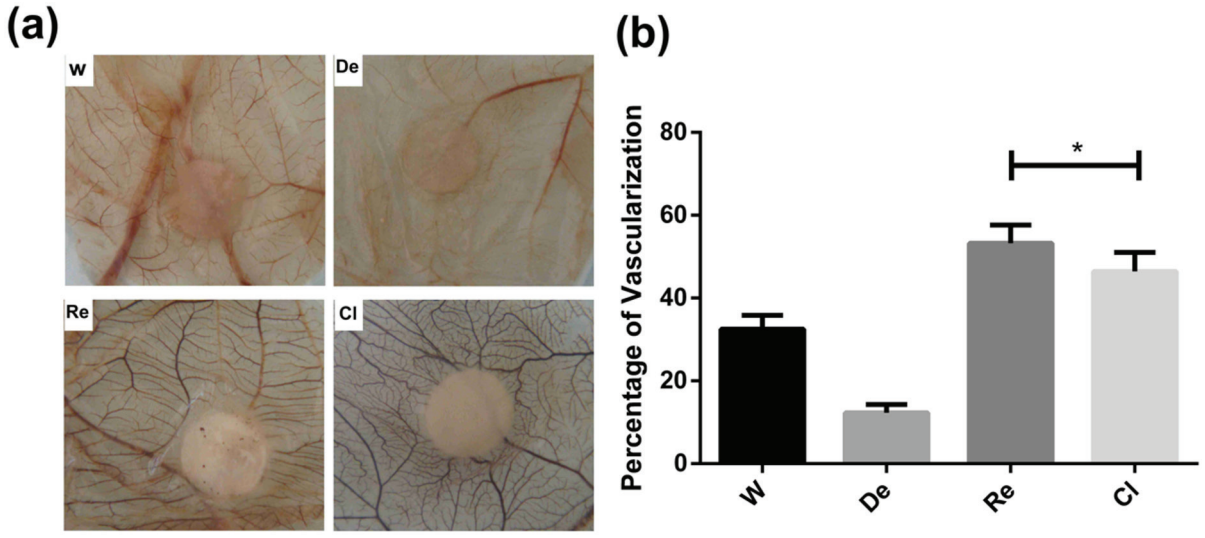

Figure 2 - (**a**) Vascular network in different controls in CAMs and (**b**) percentage of vascularization average by group: neutral control (distilled water, W); inhibitor control (dexamethasone, De); positive control (Regederm®, Re) and *C. longa* essencial oil (Cl). *Groups without significant differences among themselves. The *C. longa* essential oil showed a significant increase in the vascular network formed, compared to the neutral control and the inhibitor ($P < 0.05$), but between *C. longa* and positive control there was no significant difference ($P > 0.05$).

TABLE I
Different histological parameters observed in CAMs submitted to different treatments.

Groups	Neovascularization	Inflamatory elements	Hyperemia
Neutral control (W)	Moderate[a]	Moderate[d]	Discrete[g]
Inhibitory control (De)	Discrete[b]	Absent[e]	Absent[h]
Positive control (Re)	Accentuated[c]	Accentuated[f]	Accentuated[i]
C. longa essential oil (Cl)	Accentuated[c]	Accentuated[f]	Accentuated[i]

Same letters ($P > 0.05$); Different letters ($P < 0.05$).
All the results were compared to controls groups by Kruskal-Wallis one way ANOVA on ranks followed by multiple comparison procedure. P values less than 0.05 were considered as indicative of significance.

Uncaria rhynchophylla (Choi et al. 2005), *Salvia miltiorrhyza* (Lay et al. 2003), *Patrinia vilosa* (Jeon et al. 2010), *Pueraria Montana* (Chung et al. 2010), *Panax notoginseng* (Hong et al. 2009), *Hancornia speciosa* (Almeida et al. 2014), *Hevea brasiliensis* (Mrue et al. 2004).

As reported earlier, essential oil of *C. longa* consists of several compounds. To the best of our knowledge, there have been no reports evaluating the angiogenic activity of all essential oil compounds as a whole. However, the effect of *C. longa* isolated compounds has been widely studied. For example, there are different reports showing that curcumin exerts significant anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant and anti-infective effects (Maheshwari et al. 2006). Curcumin has also been shown to have significant wound healing properties, due to its ability to enhance granulation tissue formation, collagen deposition, tissue remodeling and wound contraction (Akbik et al. 2014).

Although many researchers have demonstrated that most pharmacological activities of *C. longa* are attributed to curcumin, this compound was found in low concentrations in *C. longa* essential oil. The results presented in this article show the

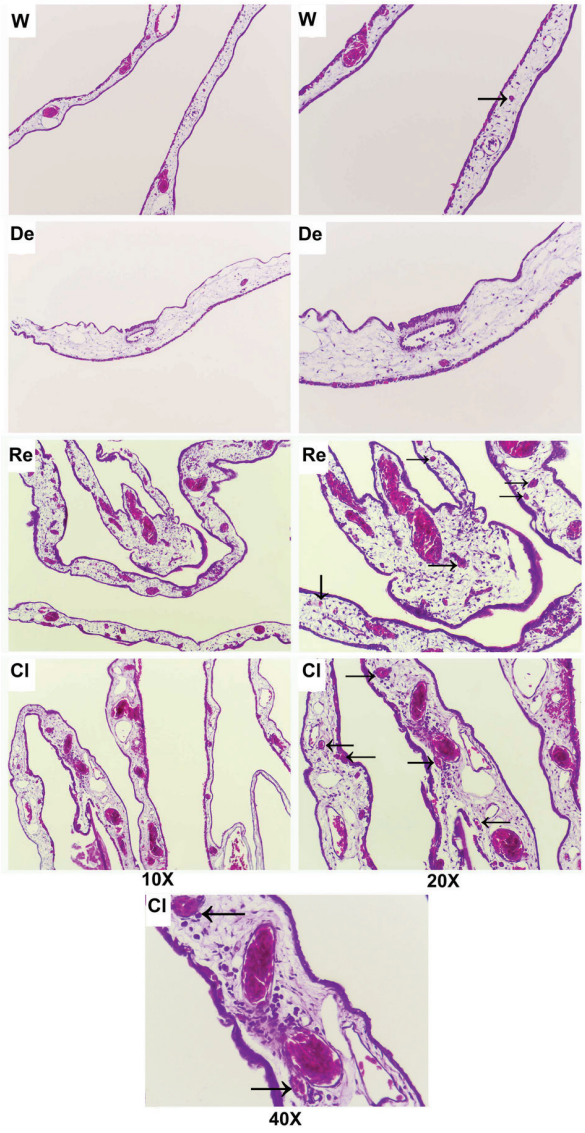

Figure 3 - Paraffin sections stained with hematoxylin-eosin with 10, 20 and 40x magnification. **W:** neutral control (distilled water); **De:** inhibitor control (dexamethasone); **Re**: positive control (Regederm®); **Cl:** *C. longa* essencial oil (Cl). The arrows show neovascularization. Inflamatory elements are visualized as purple dots. Those elements were found more frequently in Re and Cl. Hyperemia is identified by high erythrocytes concentration (Re and Cl).

angiogenic effect of *C. longa* essential oil, which could be due to activation of the inflammatory response of a specific phytoconstituent in synergy with curcumin. Other studies have shown that the synergic effect can be higher than the major compounds individually (Gill et al. 2002). This finding suggests that minority compounds may also be an important additive in the angiogenic process of *C. longa* essential oil.

CONCLUSIONS

The present study carried out photophysical and physicochemical characterization of *C. longa* essential oil as well as evaluating on its angiogenic potential. The results show that small amounts of curcumin are present in the essential oil and that the oil exhibits high thermal stability up to 90ºC. Furthermore, it was observed that curcumin is the main fluorescent component and that fluorescence emission can be attributed to the fluorescent resonance energy transfer (FRET) process. Essential oil showed considerable proangiogenic activity, which may be due to a synergic effect between its minor components. This result is an important indication that *C. longa* essential oil can be used as a compound to stimulate angiogenesis.

RESUMO

Neste estudo foram analisadas as propriedades físico-químicas e fotofísicas do óleo essencial de *Curcuma longa*, bem como seu potencial angiogênico. Os resultados obtidos mostraram que a curcumina é o principal componente fluorescente, mas está presente em pequena quantidade no óleo essencial. O método experimental da membrana corioalantóide foi empregado para avaliar a atividade angiogênica do óleo e foi observado um aumento significativo na rede vascular nos grupos submetidos ao óleo de *Curcuma longa* e controle positivo, quando comparado com os grupos controle negativo e inibidor ($P < 0,05$), mas não houve diferenças significativas entre o grupo tratado com óleo de *Curcuma longa* e o controle positivo ($P > 0,05$). A análise histológica mostrou maior neovascularização, hiperemia e elementos inflamatórios nos grupos controle positivo e *Curcuma longa* quando comparado aos demais grupos controles ($P < 0,05$), fatores característicos do processo de angiogênese. Em conclusão, o óleo de *Curcuma longa* mostrou uma considerável atividade

pró-angiogênica e pode ser um composto com potencial aplicações médicas.

Palavras-chave: atividade angiogênica, curcumina, óleo essencial, físico-química.

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