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Analysis of the Anti-inflammatory and Antioxidant Potential of the Fractions Obtained From the Ethyl Acetate Partition of the Leaves of Diplopterys Pubipetala

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Authors' contributions

This work was carried out in collaboration among all authors. Authors CAA, KTS, MCFS, SSA and SLAA performed the trial protocol, designed the study, wrote the protocol and author CAA wrote the first version of the manuscript. Author EVM performed the statistical analysis. Authors VAR, FDF and MMC managed the analyses of the study. Author CAA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The objective of this work was to analyze the anti-inflammatory, cytotoxic and antioxidant activity of the fractions of ethyl acetate from the leaves of *D. pubipetala.*
Methodology: Liquid-liquid partitioning was performed, the ethyl acetate partition was eluted in classical liquid chromatography, followed by thin layer chromatography. Antioxidant activity was performed by the DPPH photochlorimetric method and the levels of interleukin (IL-6), tumor necrosis factor α (TNF-α), interleukin-10 (IL-10) and nitric oxide (NO) were identified in supernatants of cell cultures.

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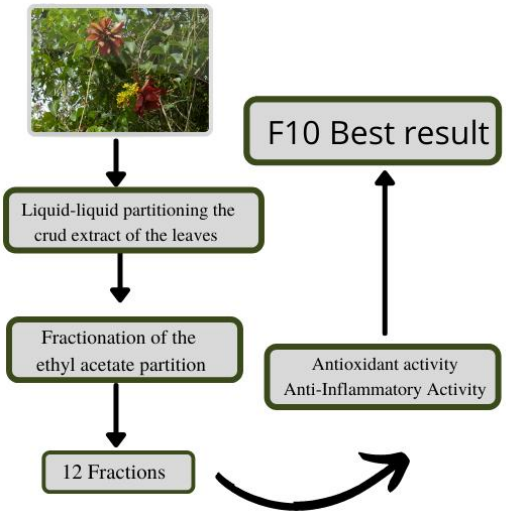
Results: Fractions F4, F5 and F10 showed antioxidant activity. The F10 fraction indicated efficient anti-inflammatory activity by the production of IL-10 and nitric oxides. Fractions F4, F5 and F9 stimulated the production of IL-6 and TNF-α.
Conclusion: These results indicate a possible synergistic action of the extract and the partition of ethyl acetate, since after fractionation the potential presented in previous studies was lower.

Graphical Abstract



Keywords: Diplopterys; nitric oxide; inteleukin-10; Interleukin-6; tumor necrosis factor-α; chromatography; antioxidant; anti-inflammatory; malpighiaceae.

1. INTRODUCTION

The Malpighiaceae family is represented by 75 genera and 1,300 species, with tropical and subtropical distribution. In the American continent, there are approximately 44 genera with about 800 species, while in the Cerrado around 22 species belonging to this family are found [1,2]. The phylogeny of this family is based on morphological analyzes of the fruit and pollen grain and on species distribution [3,4].

Due to taxonomic disparities, the genus *Banisteriopsis* (Malpighiaceae) underwent changes and its species were distributed in three genera: *Banisteriopsis*, *Diplopterys*, *Bronweia* [3]. The genus *Diplopterys* comprised only four species [5], after molecular and phylogenetic studies, *Banisteriopsis pubipetala* was included in the genus *Diplopterys* [3,6]. In morphological studies of the fruit, it was confirmed that the species *Diplopterys pubipetala* presents some developmental characteristics belonging to the genus *Diplopterys* and developmental disparities when compared to the development of *Banisteriopsis* [7]. Today, *Banisteriopsis pubipetala* and *Diplopterys pubipetala* are considered synonyms of the same species.

The species *D. pubipetala*, known as 'cipó-preto', 'cipó-de-rego', 'crista-de-galo', is a liana growing up to one and a half meters in height, which flowers in September and bears fruit from November, and is widely distributed throughout Latin America [8,9]. *D. pubipetala* is frequently found in forests and savannas of the Cadeia do Espinhaço e Planalto Central [5] and has the potential to recover degraded or disturbed areas, due to its rapid regeneration capacity [10]. The glands have a type of secretion that indicates specialization for interaction with pollinators [11,12].

Qualitative phytochemical tests performed on *D. pubipetala* indicated the presence of tannins, flavonoids, terpenoids and steroids, with emphasis on flavonoids, whose appearance in the tests was greater [12]. In the extracts of *D. pubipetala*, the presence of alkaloids, flavonoids and terpenes was identified, described in the literature as a class of secondary compounds with biological potential applicable to the treatment of several diseases [13]. The species has a high oil content in the seeds and, in studies carried out, the oil extracted from the seeds showed a predominance of unsaturated fatty

acids: palmitic, oleic, linoleic and eicosanoic, used in human food, animal feed and in the cosmetics industry [14,15]. In the crude extract of *D. pubipetala* leaves, antioxidant activity and the presence of flavonoids were identified [16]. The different partitions of *B. pubipetala* (*D. pubipetala*) extract indicated results of concentration-dependent cytotoxicity, decreased production of pro-inflammatory cytokines (TNF-α and IL-6) and increased production of IL-10 and NO. These are indications that extracts from the leaf of *B. pubipetala* (*D. pubipetala*) have anti-inflammatory potential and may exert immunoregulatory activity)] [17]. It was then sought to fractionate the ethyl acetate partition and to understand the role of these fractions in the anti-inflammatory and antioxidant activity, since the fractions indicated cytotoxic potential and stimulator of the death of cutaneous melanoma cells [18].

Antioxidant activity is conceptualized as a redox transition involving the donation of a single electron to a free radical species [19]. This activity is the basis for the maintenance of cell structure and function, being essential for biological functions such as anti-cancer, anti-inflammatory and anti-aging activities, in addition to acting in the prevention of chronic diseases such as diabetes and cardiovascular diseases [20,21].

Cytokines are small secreted proteins that are produced by almost all cells to regulate and influence the immune response [22]. The cytokines of the Interleukin-6 family have several functions in the immune system, including stimulation of B cells and induction of hepatic acute phase proteins, they also act in metabolic and hepatic functions [23]. Interleukin-10 (IL-10) is an anti-inflammatory cytokine with important immunoregulatory functions, being secreted mainly by antigen-presenting cells, such as activated T cells, monocytes, B cells and macrophages [24]. Tumor Necrosis Factor (TNF) is one of the essential cytokines during the pro-inflammatory process and plays a fundamental role in the pathogenesis of immunological disorders and in the development of tumors [25]. Nitric oxide (NO) plays an anti-inflammatory role in living systems, a role that is related to its concentration in the cellular environment, thus being considered an excellent candidate for the treatment of inflammatory diseases [26].

This work sought to evaluate the antioxidant and anti-inflammatory activity of the fractions obtained from the partition of ethyl acetate from the leaves of *D. pubipetala*, and to identify which fraction has the greatest pharmacological potential.

2. MATERIALS AND METHODS

The work was carried out from materials previously collected and extracted, with registration number in SISGEN A2EFD33.

2.1 Liquid-liquid Partitioning for Flavonoids

For the partitioning of the extract directed to flavonoids [17], the crude extract was resuspended in a mixture of ethanol: water (7:3), in the proportion of 3 g of extract to 125 mL of 70% ethanol. In the first wash of the mixture, 200 mL of hexane is added three times in a separatory funnel. In the residue of the first wash, 200 mL of ethyl acetate were added three times. The partitions were taken to an oven with air circulation at 38°C until the solvents dried [18], only the ethyl acetate partition was used for the next stages of the study.

2.2 Classical Liquid Chromatography (CLC)

The separation of the components of the *D. pubipetala* ethyl acetate partition was performed using classical liquid chromatography with increasing polarity gradient. The column used has a diameter of 2.25 cm x 80 cm height, filled with silica gel 60 for column chromatography, in the proportion of 100 mg of extract to 100 g of silica in 400 mL of hexane. The mobile phases used were: 100% hexane; hexane: ethyl acetate (Hex: AcEt) (8:2; 7:3; 1:1; 3:7; 2:8); 100% ethyl acetate; and 100% methanol in a volume of 600 mL each. Fractions were collected every 10 mL in test tubes with the same volume. The column flow rate was 2.6 mL per minute.

2.3 Thin Layer Chromatography (TLC)

The separation of the partition components carried out in Classical Liquid Chromatography (CLC) was followed by Thin Layer Chromatography (TLC). Chromatographic plates 0.25 mm thick and mobile phases of hexane and acetate (7:3; 1:1; and 3:7) were used. Chromatographic plates were revealed by spraying sulfuric vanillin (6 g of vanillin, 100 mL of ethanol and 1 mL of PA sulfuric acid).

The fractions resulting from this procedure were stored in glass flasks, named in the order of elution, and placed in an oven with air circulation at 38°C for the drying of the solvents. After drying the solvents, each fraction was weighed and the yield was calculated.

2.4 Antioxidant Activity

The evaluation of the antioxidant activity of the fractions was measured by the photocolorimetric method with DPPH (2,2 Diphenyl1-1-Pricryl-hydrazyl) [27].

The DPPH stock solution was prepared at a concentration of 40 µg/mL, the stock solutions of the fractions were prepared at 1000 µg/mL (30) and both were diluted in methanol. In separate test tubes, 3 mL of DPPH and 0.5 mL of solutions were pipetted from each sample at concentrations of 250, 500, 750 and 1000 µg/mL. Then, these solutions were kept protected from light for 30 min and then read in a spectrophotometer (SHIMADZU – UV-VIS 2550) at a wavelength of 517 nm. For the negative control, 3 mL of DPPH and 0.5 mL of methanol are pipetted, without the addition of extracts. The positive control was made from gallic acid, a stock solution of gallic acid at 80 µg/mL was prepared, dilutions of 10, 30, 50 and 80 µg/mL were made and 1000 uL of DPPH at 40 µg/mL were added. The solution was kept protected from light and then read in a spectrophotometer at 517 nm.

The average of the results of the samples and of the positive control was used for the calculations of percentage of antioxidant activity through the formula:

$$\{(AbsCont-AbsAmos)\div AbsCont\}\times 100$$

Where: AbsCont represents the absorbance value of the negative control; AbsAmos represents the absorbance value of the sample [28].

From the values of percentage of antioxidant activity, a graph was constructed in Microsoft Excel 2016 relating the percentage of antioxidant activity with the concentration of the samples (250, 500, 750 and 1000 µg/mL). Based on this graph, linear regression was performed and the EC_{50} was calculated from the generated equation for the samples and positive control. This calculation estimates the sample's ability to capture 50% of the free radicals in the DPPH solution.

Antioxidant Activity indices were also calculated through the equation:

IAA = Final concentration of DPPH• (µg/mL)/EC_{50} (µg/mL) [29].

2.5 Cell Culture

Embryonic fibroblast cells from 3T3 mice were cultured in RPMI-1640 culture medium (Sigma Aldrich, USA) and supplemented with 10% (v/v) fetal bovine serum (Invitrogen Co Ltd, USA), 100 IU penicillin/mL and 100 µg streptomycin/mL (Sigma Aldrich, USA). Cells were grown in 75 cm^2 flasks and incubated at 37°C in a humidifier with 5% CO_2.

2.6 Quantification of Cytokines interleukin-6, Tumor Necrosis Factor-α and Interleukin-10

Stock solutions of 2000 µg/mL of D. pubipetala fractions were prepared in sterile culture medium (RPMI). In the tests, the concentrations used were: 6.25; 12.5; 25; 50; 100; 150; 200 and 400 µg/mL, the choice of concentrations used were based on the results of the MTT (Thiazolyl Blue Tetrazolium Bromide) assays previously carried out by the research group [17].

Levels of IL-6, TNF-α, and IL-10 were estimated using the commercially available high-sensitivity enzyme-linked immunosorbent assay kit, ELISA, (Enzo Life Sciences, USA) according to the manufacturer's instructions. All assays were performed in triplicate. To perform the ELISA test, 3T3 cells were seeded at 5.0×10^5 cells/well in 24-well plates. 24 h after seeding the cells, they were treated with 250 µg/mL of fractions F4, F5, F6, F9, F10 for 30 min. After 24 h, cells from the supernatant were obtained by centrifuging 413 g, 10 min, and stored at −80°C.

2.7 Quantification of Nitric Oxide (NO)

Were seeded 3T3 cells at 5.0×10^5 cells/well in 24-well plates. Twenty-four hours later, the cells were treated for 30 min with 250 µg/mL of the different fractions. After 24 h, supernatants were obtained by centrifuging 413 g, 10 min and NO production were measured using the Griess reaction. A comparison of 100 µL aliquots of culture supernatant with serial dilutions of $NaNO_2$

(from 7.81 mM to 1000 mM) was performed. To this, an equal volume of Griess reagent (0.1% N-1-naphthylethylenediamine in H_2O + 1% sulfanilamide in 2.5% H_3PO_4) was added and then incubated at room temperature for 10 min and at reading performed at 540 nm [30].

2.8 Statistical Analysis

All results were analyzed by ANOVA and Tukey Test on GraphPad Prism version 9.00 for Windows (USA). P <0.05 considered as statistical significance.

3. RESULTS AND DISCUSSION

From the chromatographic profile of the ethyl acetate partition, 12 different fractions were obtained, detailed in Table 1. The fractions used in the tests were fractions F4, F5, F6, F9, F10. The choice of these fractions was due to results observed for anticancer activity in cutaneous melanoma cells [18].

3.1 Antioxidant Activity

The crude extract of the leaves of *D. pubipetala*, indicated antioxidant potential, with EC_{50} 25.2 µg/mL, this activity is related to the presence of flavonoids in the leaves of the species (Sacramento et al 2020). In the partition of ethyl acetate (EtOAc) in species of *Byrsonima*, a genus close to *Diplopterys*, EC_{50} was obtained in the following values: for the species *Byrsonima intermedia* 21.66 µg/mL, *Byrsonima coccolobifolia* 27.57 µg/mL, *Byrsonima verbascifolia* 23.66 µg/mL, *Byrsonima sericea* 11.56 µg/mL [31]. These values are close to those obtained in *D. pubipetala* (Table 2), which indicates that the species object of study has potential for antioxidant activity. Of the fractions obtained, the best result was observed in the F10 fraction (70.69 µg/mL).

For the antioxidant activity index, samples F4, F5 and F10 showed values of 0.432, 0.458, 0.565, respectively, which indicates moderate activity [29].

3.2 Anti-inflammatory Activity

During the inflammation process, defense cells B, T lymphocytes and macrophages stimulate the production of IL-6. TNF-α is also produced and it acts on endothelial changes. When the inflammatory reaction becomes exacerbated, the macrophages produce IL-10 to mitigate the uncontrolled production of inflammatory cytokines during the infection [29,32].

Table 1. Fractions obtained and yield %

Fraction name / tubes collected	CLC mobile phase	Yield (%)
F1 (1 to 62)	Hex 100%	0.9
F2 (1 to 40)	Hex:EtOAc 8:2	7.96
F3 (1 to 10)	Hex:EtOAc 7:3	5.11
F4 (11 to 25)	Hex:EtOAc 7:3	7.92
F5 (26 to 40)	Hex:EtOAc 7:3	7.38
F6 (1 to 16)	Hex:EtOAc 1:1	10.02
F7 (17 to 33)	Hex:EtOAc 1:1	7.51
F8 (34 to 36)	Hex:EtOAc 1:1	7.73
F9 (1 to 41)	Hex:EtOAc 3:7	1.08
F10(1 to 40)	Hex:EtOAc 2:8	1.37
F11 (1 to 55)	EtOAc 100%	0.83
F12 (1 to 50)	Methanol100%	14.40

Abbreviations in the table: Hexane (Hex), Ethyl Acetate (EtOAc)

Table 2. Values of the concentration equivalent to 50% (EC_{50}) and the antioxidant activity index of fractions F4, F5, F10

Fractions	EC_{50} µg/mL	R^2	Antioxidant Activity Index
F4	92.43	0.996	0.432
F5	87.20	0.980	0.458
F10	70.68	0.980	0.565
Gallic Acid	3.29	0.987	12.146

The IL-6 production values are detailed in the graphic of Fig. 1-a, the F10 fraction induced greater IL-6 production (266.536 pg/mL), and the F9 fraction was not considered statistically significant. The F5 fraction induced the production at a concentration of 183.527 pg/mL, while the F4 and F6 fractions induced values close to the control, 120.622 pg/mL and 132.313 pg/mL respectively, in this context the F4 and F6 fractions obtained less pro-inflammatory stimulation. In studies carried out on the ethyl acetate partition of *D. pubipetala*, it showed low IL-6 values, being about 14 pg/mL at a concentration of 100 µg/mL, these values are consistent with results obtained in plants belonging to the same family, such as *Malpighia emarginata*, for which there were results close to 9 pg/mL in an aqueous extract at 12.5 µg/mL, the methanolic extract of *Lophanthera lactescens* showed about 500 pg/mL at a concentration of 100 mg/kg [17,33,34].

The values obtained for TNF-α production are detailed in Fig. 1-c. The F10 fraction induced the highest production of TNF-α (638.586 pg/mL), the F5 fraction was the second biggest stimulator of production (251.686 pg/mL), followed by the F4 fraction (189.084 pg/mL). Fractions F6 and F9 produced the lowest values, closer to the control (155.118 pg/mL and 144.927 pg/mL respectively) indicating that they have a low pro-inflammatory action. EtOAc partitioning from *D. pubipetala* induced the production of about 14 pg/mL to 100 µg/mL of TNF-α, indicating a small inflammatory potential [17]. In species of the genus close to *Diplopterys*, of *Byrsonima* gender, indicated the production of TNF-α between 60 to 65 ng/mL in extracts at 50 µg/mL, so the fractions of *D. pubipetala* showed higher production of TNF-α than similar species [31].

IL-10 production is detailed in Fig. 1-b. The F10 fraction induced higher production of IL-10, 629.703 pg/mL. Fractions F4 and F9 induced a lower production, obtaining values closer to the control, 229.895 pg/mL and 235.083 pg/mL respectively. The F5 fraction indicated the second highest production, 378.080 pg/mL, followed by the F6 fraction, 290.531 pg/mL. The EtOAc partition indicated IL-10 production capacity, (39 pg/mL in extracts at 100 µg/mL). In *Malpighiae marginata*, the production of IL-10 by the aqueous extract was around 7 pg/mL in the stratum of 12.5 µg/mL. These results indicate that the production of IL-10 in *D. pubipetala* is consistent with species of the family [17,33].

NO acts as a mediator in several functions of the immune system, among them the function of inhibiting the growth of microbial pathogens and protecting tissues damaged by acute systemic inflammation [35]. The production of NO by 3T3 cells, after exposure to the fractions, is detailed in Fig. 2. Is possible to observe that only the F10 fraction obtained a statistically significant value (130.453 µM/mL). *D. pubipetala* ethyl acetate partitioning stimulated the production of 29 µM to 100 µg/mL of NO. [17]. The high levels of NO, associated with the strong presence of IL-10 indicate that its action is related to anti-inflammatory activity.

This suggests a possible synergistic effect of the extract. The substances that constitute an extract can be divided into active substances, co-effectors and matrix formers, and the interaction between them can protect the active substances from decomposition [36]. The anti-inflammatory and antioxidant effect can be exerted by different types of phytochemical combinations, and five general mechanisms for the anti-inflammatory action have been proposed: increased availability of phytochemicals in the extract, increased antioxidant capacity, interaction with the intestinal microbiota and targeting of signaling pathways [37]. The extracts may have biological activities due to the presence of several phenolics in natural proportions and their complementary activities [38]. In addition, it is possible to verify the existence of a cytotoxic or undesirable effect when a major component of the extract is tested alone [36].

The quantification of cytokines was determined by the ELISA method. Each experiment was performed in triplicate. Cells were treated with 250 µg/mL D. pubipetala fractions. *P = 0.05 when compared to the negative control.

The experiments were performed in splice. P= 0.05 when compared to the negative control.

It was observed that the F10 fraction was more prominent in the anti-inflammatory action, as it stimulated the production of the cytokine IL-10 and nitric oxide, both of which have a mediating capacity in the reduction of the inflammatory processes of the immune system. Despite this potential, F10 also indicated the ability to stimulate the production of cytokines related to the increase in the inflammatory process, such as TNF-α. The production of this cytokine may be related to the ability of this fraction to potentiate the death of cutaneous melanoma

cells, since the decrease in the production of TNF-α may be associated with the progression of this disease [18,39]. More studies should be carried out to identify the molecule present in the fraction that enabled its anti-inflammatory activity, as well as to identify the lethal concentration.

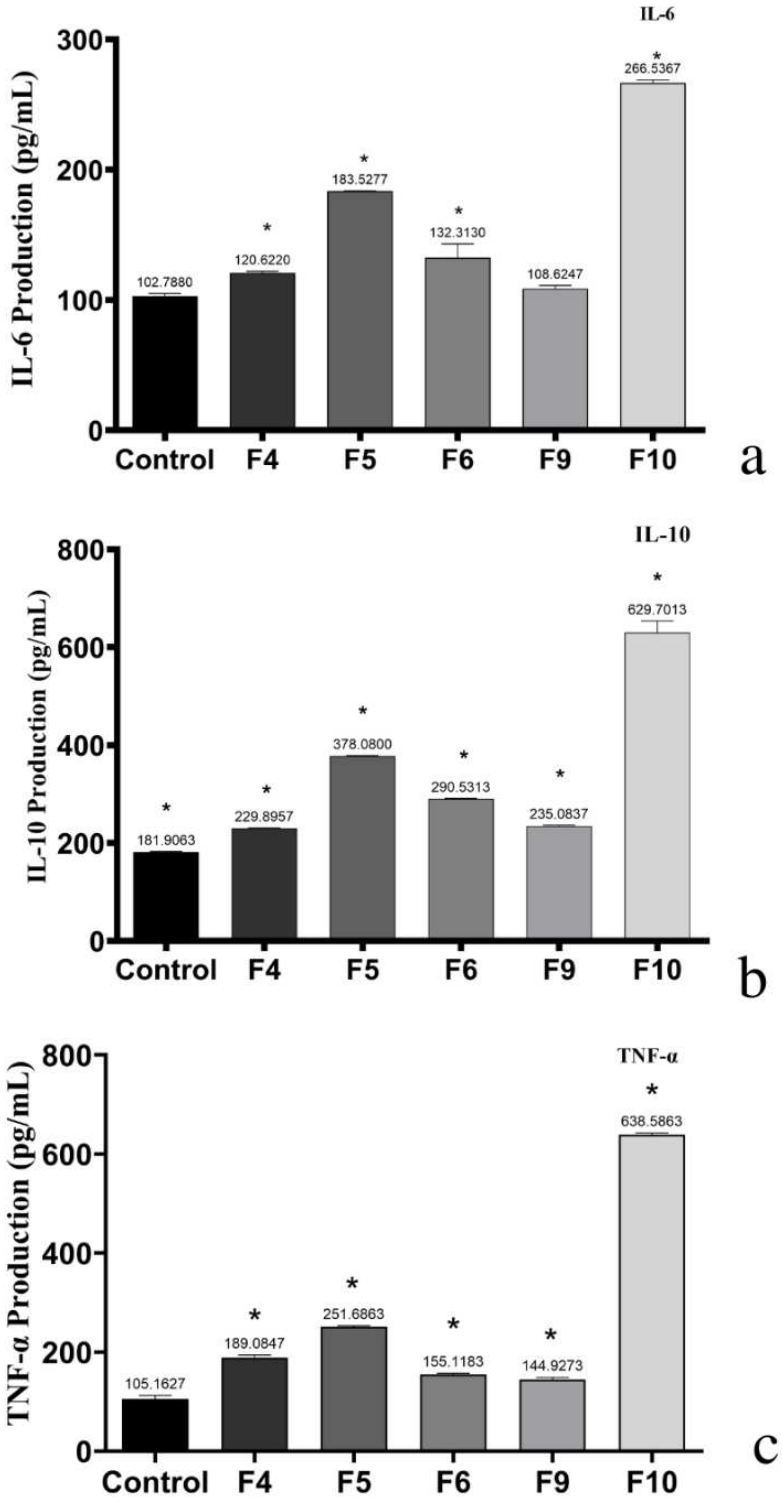


Fig. 1. Effect of D. pubipetala fractions on the production of cytokine IL-6, IL-10 and TNF-α

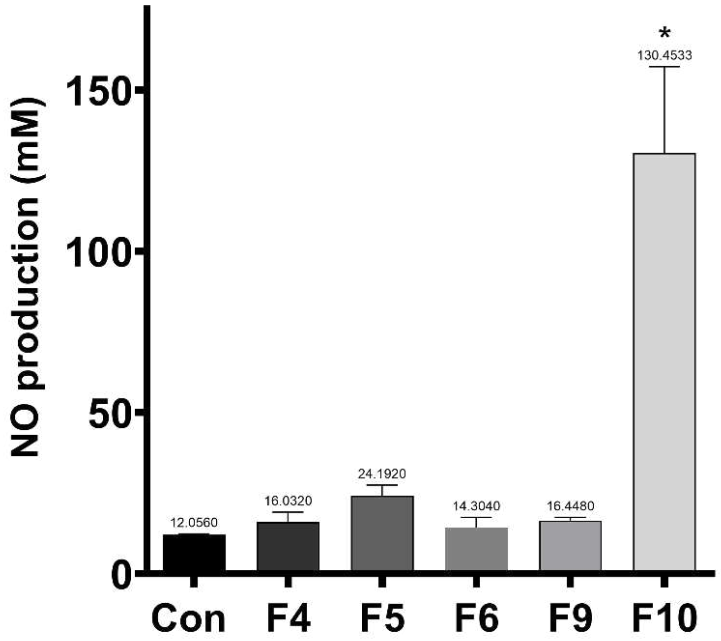


Fig. 2. Effect of D.pubipetala extract on nitric acid production. NO production in 3T3 cells was determined by the Griess reaction after 24 h of incubation with 250 µg/mL of the fractions

In studies carried out on the partition of ethyl acetate, *D. pubipetala* showed anti-inflammatory potential, from the stimulation of the production of IL-10 and nitric oxide [17]. This may have occurred due to the different concentrations used, and the increase in the dose may have reduced the inflammatory capacity of the fraction and stimulated a cytotoxic action [40].

In studies carried out by our research group, it can be observed that the plant has molecules belonging to the phenolic group, capable of having anti-inflammatory and antioxidant responses [17]. Phenolic compounds are compounds derived from a common building block of the carbon skeleton, capable of acting as antioxidants [41]. Commonly known for promising results in relation to antimicrobial activity, for phenolic compounds, anti-inflammatory activity can be observed due to the action against irritant and the elimination of reactive oxygen species (ROS), they are effective in experimental inflammation, since the prevention production of reactive oxygen species by cells of the immune system may be related to the inhibition of actions [42].

In a study carried out, in the crude extract of *D. pubipetala* leaves, the potential for antioxidant activity was verified, with an EC_{50} value of 25.2 µg/mL, which may be related to the presence of phenolics and flavonoids present in this extract [16]. As for the isolated fractions, the antioxidant activity did not stand out, maintaining a relatively high value of EC_{50} for the fractions F10, F9 and F4, which may indicate that the antioxidant action of the extract comes from the joint action of the compounds belonging to the general class of phenolics.

4. CONCLUSION

From the results obtained, it was observed that, from the fractionation of the crude extract of *D. pubipetala*, the F10 fraction showed better antioxidant activity, but also showed a high value of EC_{50}, when compared with the values of the crude extract. The study also indicated that the fractions showed anti-inflammatory activity, due to increased production of IL-10 and nitric oxide, in 3T3 mouse fibroblast cells, after exposure to the fractions. Especially the fraction F10, which substantially increased NO values. However, the fractions also stimulated the production of pro-inflammatory cytokines IL-6 and TNF-α, a fact that may be related to the concentration of the administered dose and possibly to the ability of these cytokines to induce the death of cutaneous melanoma cells observed in the work of the group. Finally, these results indicate a possible

synergistic action of the extract and the partition of ethyl acetate, since after fractionation the potential presented in previous studies was lower.

CONSENT

It's not applicable.

ETHICAL APPROVAL

It's not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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