

Full Research Paper

## Quantification of Phenolic Constituents and Antioxidant Activity of *Pterodon emarginatus* Vogel Seeds

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**Abstract:** In the present study the phenolic (Folin-Dennis) and flavonoid (colorimetric assay) constituents and the antioxidant activity of *Pterodon emarginatus* seeds were investigated in several samples prepared with different extraction procedures: essential oil (EO) using a Clevenger-type apparatus; hexanic (HF), ethyl acetate (EAF), buthanolic (BF) and methanolic (MF) fractions using Soxhlet extraction, and extracts (1 g/extract) obtained from different methods: reflux 80°C/30 min, ultrasound/30 min, static maceration/48 h and heating plate 100°C/45 min. These extracts were prepared using water or ethanol/water at 30:70 v/v, 50:50 v/v or 70:30 v/v. Antioxidant activity [2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH)] was tested only in the fractions obtained from Soxhlet extraction. The extract obtained from reflux using ethanol/water (70:30, v/v) showed the highest phenolic constituents level. The EAF, BF and MF showed DPPH scavenging activities with IC<sub>50</sub>=163.22, 18.89 and 10.15 µg/ml, respectively.

**Keywords:** *Pterodon emarginatus*, Leguminosae, phenolic constituents, DPPH.

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## 1. Introduction

Oxidation is a basic part of the aerobic life and of our metabolism. Thus radicals are produced either naturally or by some biological dysfunction. Unpaired electrons which are centered in atoms of oxygen or nitrogen are called reactive oxygen species (ROS) or reactive nitrogen species (RNS) [1-4] and its excess has a harmful effect, such as the peroxidation of the membrane lipids, aggression to tissue proteins and membranes, on damage to DNA and enzymes [5]. Therefore, they can be related to some pathologies, such as arthritis, hemorrhagic shock and coronary diseases, cataract, cancer, AIDS as well as age-related degenerative brain disorders [6].

Currently, there is a great interest in the study of antioxidant substances mainly due to the findings of the free radicals' effects in the organism. The phenolic constituents found in vegetables have attracted considerable attention for being the main components of antioxidant activity, in spite of not being the only ones. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelation potential. The antioxidant activity of phenolics play an important role in the adsorption or neutralization of free radicals [7].

The genus *Pterodon* (Leguminosae) comprises five species native to Brazil: *Pterodon abruptus* Benth., *Pterodon apparicioi* Pedersoli, *Pterodon polygalaeflorus* Benth., *Pterodon pubescens* Benth. and *Pterodon emarginatus* Vogel. *Pterodon emarginatus* Vog., known in Brazil as sucupira-branca or faveiro. It is a native aromatic tree reaching 5-10 m in height distributed throughout the central region of Brazil (Goiás, Minas Gerais and São Paulo) [8]. Hydroalcoholic infusions of seeds are used in folk medicine for their anti-rheumatic, analgesic and anti-inflammatory properties [9-11].

Chemical studies on *Pterodon* have shown the presence of alkaloid compounds in the bark [12], isoflavone and some triterpenes in the wood [13], and diterpenes [14-15] and isoflavones in the seed oil [16]. The diterpene 14,15-epoxygeranylgeraniol and some derivatives isolated from *P. pubescens* have been associated with protective activity against the penetration of cercarias of *Schistosoma mansoni*, a typical tropical disease [17]. The furan-diterpene 6 $\alpha$ , 7 $\beta$ -dihydroxyvouacapan-17 sodium-oate, isolated from the *P. polygalaeflorus* Benth's fruit oil, showed anti-inflammatory activity in the paw edema produced by carrageenin [11, 18-19].

Jantová *et al.* (2000) found that the plants possess significant cytotoxic and antibacterial activities [20-21], but the chemical composition of the extracts were not elucidated. Only a limited number of papers have described the various flavonoid, amino-containing, triterpene, steroid, megastigmane, saccharide, organic and fatty acid compounds found therein [22]. Due to these findings, we have focused on establishing a relationship between the total phenolic content and antioxidant activity of seeds of *P. emarginatus*.

## 2. Results and Discussion

The total phenolic content of *P. emarginatus* seeds is shown in Table 1. The results demonstrated that the reflux and heating plate were more efficient for polyphenols extraction showing the efficiency of heat in the extraction of these compounds. However, comparing the heating plate with reflux, using ethanol/water (70:30, v/v) as solvent, a smaller concentration of phenolic constituents was obtained in

heating plate method, suggesting that this procedure is involved in the degradation of some polyphenolic constituents.

The temperature during the drying and heating process affects compound stability due to chemical and enzymatic decomposition, losses by volatilization or thermal decomposition; these latter have been suggested to be the main mechanisms causing the reduction of polyphenol contents. However, the total polyphenols in extracts obtained of *P. emarginatus* increased from 781.60 (plate heating) to 852.60 mg/100g (reflux), this is most likely attributed to the formation of phenolic substances under milder heating temperature whereas polyphenols are degraded at elevated heating temperatures, such as plate heating. The formation of phenolic compounds during the heating process might be due to the availability of phenolic molecules' precursors, by non-enzymatic interconversion amongst phenolic molecules subjected to the effects of external factors, such as temperature [23]. Thus, the plant composition and the degree of heating could be important factors contributing to high total polyphenol content. However, further investigation is needed to truly explain this phenomenon.

Solvent extraction is frequently used for isolation of antioxidants and both extraction yield and antioxidant activity of extracts are strongly dependent on the solvent, due to the different antioxidant potentials of compounds with different polarity [24-25]. In this work, the extraction made under reflux using ethanol/water (70:30, v/v) presented the highest polyphenol level, and was considered the ideal method for the extraction of such constituent. It leads to the conclusion that the increase in the amount of ethanol in the solvent contributes to the extraction of tannins of higher molecular weight [26].

It was not possible to detect the phenolic constituent in essential oil (EO) and hexanic (HF), ethyl acetate (EAF), buthanolic (BF) and methanolic (MF) fractions of *P. emarginatus* seeds.

**Table 1.** Phenolic constituents of *P. emarginatus* seeds submitted the different processes of extraction<sup>#</sup>.

Extraction procedures	Phenolic constituent (mg/100g)			
	Water	Ethanol/water (30:70, v/v)1	Ethanol/water (50:50, v/v)	Ethanol/water (70:30, v/v)2
Reflux a	ND	5.20 ± 0.25	600.30 ± 2.51	852.60 ± 2.51
Maceration	ND	ND	ND	501.00 ± 1.73
Ultrasound	ND	ND	ND	603.00 ± 2.08
Plate of heating b	113.00 ± 1.00	332.60 ± 2.51	536.30 ± 1.52	781.60 ± 1.52

<sup>#</sup> Data are expressed as the mean ± standard deviation (n=3). Statistical significance was calculated by ANOVA followed by Tukey test. Letters indicate statistical differences between lines. Numbers indicate statistical differences between columns. <sup>a, b, 1, 2</sup> all methods show statistical differences.

The levels of flavonoids are shown in Table 2. In the heating plate method the level of flavonoids was the smallest amongst the tested methods, what leads to the conclusion that under such conditions the flavonic constituents in this sample may be degraded.

**Table 2.** Flavonoids levels of *P. emarginatus* seeds submitted to the different processes of extraction<sup>#</sup>.

Extraction Method	Flavonoids (mg/100g)			
	Water <sup>6</sup>	Ethanol/water (30:70, v/v) <sup>1</sup>	Ethanol/water (50:50, v/v)	Ethanol/water (70:30, v/v)
Reflux	132.00 ± 1.00	122.00 ± 2.00 <sup>a</sup>	106.06 ± 3.05 <sup>b,2</sup>	120.30 ± 1.53 <sup>a,4</sup>
Maceration	110.00 ± 1.00 <sup>d</sup>	103.30 ± 2.3 <sup>c</sup>	110.60 ± 1.15 <sup>d,2</sup>	123.00 ± 1.73 <sup>4</sup>
Ultrasound	101.70 ± 1.53 <sup>f</sup>	92.60 ± 2.5 <sup>e</sup>	96.30 ± 1.53 <sup>e</sup>	131.30 ± 1.53
Plate of heating	68.00 ± 1.00	67.00 ± 1.00	73.70 ± 1.15 <sup>g,3</sup>	68.00 ± 1.00 <sup>5</sup>

# Data are expressed as mean ± standard deviation (n=3). Statistical significance was calculated by ANOVA followed by Tukey test. Letters indicate statistical differences between lines. Numbers indicate statistical differences between columns.

<sup>a</sup> p<0.05 compared to water and ethanol/water (50:50, v/v); <sup>b</sup> p<0.05 compared to water; <sup>c</sup> p<0.05 compared to water, ethanol/water (50:50, v/v) and ethanol/water (70:30, v/v); <sup>d</sup> p<0.05 compared to ethanol/water (30:70, v/v) and ethanol/water (70:30, v/v); <sup>e</sup> p<0.05 compared to water and ethanol/water (70:30, v/v); <sup>f</sup> p<0.05 compared to ethanol/water (70:30, v/v); <sup>g</sup> p<0.05 compared to water, ethanol/water (30:70, v/v) and ethanol/water (70:30, v/v); <sup>1, 6</sup> all methods show statistical differences; <sup>2</sup> p<0.05 compared to ultrasound and plate of heating; <sup>3</sup> p<0.05 compared to ultrasound, maceration and reflux; <sup>4</sup> p<0.05 compared to ultrasound and plate of heating; <sup>5</sup> p<0.05 compared to ultrasound, maceration and reflux.

Water is a polar extractor, so it will extract polar constituents, such as heterosides. In this work, when water was used as liquid extractor, the level of flavonoids was higher, suggesting that these flavonic constituents might be in the heteroside form since previous studies showed that flavonoids can be found in nature in the free state or in the form of glycosides [27-29].

The fractions obtained by Soxhlet from *P. emarginatus* seeds, presented flavonoids: 2.92 mg/100 mg, 3.16 mg/100 mg and 3.01 mg/100 mg, respectively EAF, BF and MF fractions. It EO and HF was not possible to determine flavonic constituents.

The antioxidant activity of *P. emarginatus* was evaluated in the EO and fractions obtained from Soxhlet by their ability to scavenge DPPH free radicals. The radical scavenging activity of the compounds can be measured as a decolorizing effect following the trapping of the unpaired electrons of DPPH. A lower value of IC<sub>50</sub> indicates a higher antioxidant activity. The BF and MF demonstrated scavenging activity of DPPH radicals with IC<sub>50</sub> values of 18.89 µg/ml and 10.15 µg/ml, respectively. The EAF showed a smaller antioxidant activity (IC<sub>50</sub> = 163.22 µg/ml). However, the EO and HF did not demonstrate antioxidant activity at all tested concentrations. Ascorbic acid and butylated hydroxytoluene (BHT) produced IC<sub>50</sub> values of 2.50 µg/ml and 7.58 µg/ml, respectively.

Based on previous data, it is possible that the powerful antioxidant activity of polar extracts is given by the presence of the substance with hydroxyls [30-31]. In this context, flavonoids possess an ideal

structure for the scavenging of free radicals since they present a number of hydroxyls acting as hydrogen-donators which makes it an important antioxidant agent [7, 32].

The key role of phenolic compounds as scavengers of free radical is emphasized in two important reports [33-34]. Antioxidative properties of essential oils and various extracts from many plants are of great interest in both academia and the food industry since their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidants by natural ones. In this respect, studying the medicinal species may be of interest since their bioactive properties could be lost forever without being tapped. The results of this work indicate that the EAF, BF and MF obtained from *P. emarginatus* seeds showed capacity to donate hydrogen, therefore they present scavenging activity of DPPH. This activity might be due to the presence of flavonic constituents detected in the samples.

### 3. Conclusions

This study reports for the first time the antioxidant activity and total phenolic content in *P. emarginatus* seeds. Buthanolic (BF) and methanolic (MF) fractions exhibited a marked scavenging effect on DPPH radical. Chemical elucidation of the extracts' contents and fractions of *P. emarginatus*, should be the main objective of further investigations. This should lead to the structural identification of the active metabolites and the determination of their antioxidant profile in pure form.

### 4. Experimental

#### General

Plant material (seeds) of the specie *Pterodon emarginatus* was collected in September 2006 at the city of Três Marias (Minas Gerais, Brazil) and authenticated by Dra. Fátima Regina Gonçalves Salimena from the Department de Botany, UFJF, Brazil. A voucher specimen (N° 48.077) has been deposited at the CESJ Herbarium of the Federal University of Juiz de Fora (Minas Gerais, Brazil).

The seeds of *Pterodon emarginatus* were triturated (30 g) and hydrodistilled in a Clevenger-type apparatus. After 2 h distillation, the essential oil (EO) was collected as a film floating on the surface of the water. The samples were sealed and kept in dark glass vials in the refrigerator at 4°C for further analysis.

Thirty grams of seeds were triturated with the aid of pruning shears and submitted to extraction using a Soxhlet extraction device with hexane, ethyl acetate, buthanol and methanol until reaching the exhaustion of the drug. The fractions were subjected to rotary evaporation, until complete elimination of the solvent, four fractions of different polarities were obtained: hexanic (HF), ethyl acetate (EAF), buthanolic (BF) and methanolic (MF).

So as to quantify the phenolic and flavonoid constituents were used fractions obtained by Soxhlet (described above) and several extracts (1 g/extract) obtained by four different methods of extraction: reflux 80°C during 30 min, ultrasound for 30 min, static maceration during 48 h and heating plate 100°C for 45 min. These extracts were prepared using 30, 50 and 70% ethanol and distilled water as solvent. These extracts were prepared using: A (water), B [30:70, v/v ethanol/water], C [50:50, v/v ethanol/water] and D [70:30, v/v ethanol/water] as extraction media.

A spectrophotometric method explained AOAC [35] was adapted for the phenolic content assay. Extracts or fractions thereof (described above), were dissolved in methanol to obtain a concentration of

0.5 mg/ml. Folin–Dennis reagent (100 µl) was added to a test tube containing the extracts or fractions (10 µl). Contents were mixed and a saturated sodium carbonate solution (200 µl) was added to the tube. Volume was adjusted to 2 ml by the addition of 1.69 ml of distilled water and the contents were mixed vigorously. Tubes were allowed to stand at room temperature for 25 min and then centrifuged for 5 min at 2435 x g. Absorbance of the supernatant was read at 760 nm. Blank samples of each extract or fraction were used for background subtraction. Tannic acid was used as standard for the construction of the calibration curve (2-10 µg/ml). The assay was carried out in triplicate.

The procedure used for the quantification of flavonoids is based on the reaction between the flavonoids and aluminum chloride forming a complex with a yellow color that can be measured in a spectrophotometer at a wavelength of 420 nm [36]. Rutin was used as standard for the construction of the calibration curve (2-30 µg/ml). For the quantification of the flavonoids content in the extracts or fractions thereof, aliquots of 4 ml of chloroform and 6 ml of distilled water were added to 10 ml of the previously obtained samples. The resulting solutions were mixed and centrifuged for 3 min at 2435 x g. Two milliliters of the aqueous phase was diluted to 25 ml with 10 ml of the reagent (pyridine, distilled water and aluminum chloride solution 17:80:3, v/v), 12.4 ml of a solution composed of water and dimethyl sulfoxid (1:2, v/v) and 0.6 ml of glacial acetic acid and, soon after vortexing the reaction mixture, the tubes were placed in the dark for 15 min and the absorbance was measured at a wavelength 420 nm against the reagent blank. The construction of the calibration curve and the preparation of the sample solutions of the hydroalcoholic extracts for reading were done in triplicate.

The radical scavenging activity were determined by the 2,2- diphenyl-2-picrylhydrazyl hydrate (DPPH) (Sigma-Aldrich Chemie, Steinheim, Germany) method [37]. The molecule DPPH characterized as stable-free radical by virtue of the delocalization of the spare electron over the molecule; this delocalization produces a deep violet color, characterized by an absorption band in ethanol or methanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substance, that can donate a hydrogen atom, this gives rise to the reduced form (diphenylpicrylhydrazine), with the loss of violet color.

An aliquot (0.5 ml) of ethanol solution containing different amounts of the essential oil (OE) and fractions (HF, EAH, BF and MF) obtained from seeds of *Pterodon emarginatus* (0.97-250 µg/ml) was added to 1.5 ml of daily prepared ethanol DPPH solution (0.05 mM). The optical density change at 517 nm was measured 30 min later by a spectrophotometer. A blank was used to remove the influence of the color of the samples. An ethanolic solution of DPPH was used as negative control. Ascorbic acid and butylated hydroxytoluene (BHT) were used as reference drugs, at the same concentrations (0.97-250 µg/ml) as were used for the samples. Results were expressed as mean inhibiting concentration (IC<sub>50</sub>). IC<sub>50</sub> parameter is defined as the concentration (µg/ml) of substrate that causes 50% loss of DPPH activity (color) and it was calculated by using the following equation:  $IC_{50} (\%) = 100 \times (A_0 - A_s)/A_0$ , where  $A_0$  and  $A_s$  are the values for the absorbance of the negative control and the absorbance of the sample, respectively. Tests were carried out in triplicate.

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