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Phenolic compounds, flavonoids and antioxidant activity of leaves, flowers and roots of white-weed

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ABSTRACT

Ageratum conyzoides (mentrasto or white-weed) has invaluable socio-economic-cultural importante being used as unconventional vegetable in traditional cuisine of Minas Gerais state, Brazil. In folk medicine it is used as a purgative, febrifuge, antiemetic and against colic, ulcers and uterine problems. The objective of this study was to quantify the concentration of phenolic compounds, flavonoids and antioxidant activity in ethanol extracts of white-weed, as a step towards analytical and bioanalytical validation of its use as phytotherapic and as functional food. Thus, analysis of the levels of phenolic, flavonoids and antioxidant activity (AAT %) were made in ethanol extracts of leaves (EF), flowers (EFL) and roots (ER) of white-weed. Measuring phenolic compounds in EF, ER and EFL, was carried out by the Folin-Ciocalteu method. The content of flavonoids in the extracts was determined using vanillin in acid medium. For the antioxidant evaluation we used the methodology of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging method. The experimental design was randomized blocks design, all analyzes being performed with four replications. Data were submitted to analysis of variance and to test at 1% significance. ER had a higher concentration of phenolic compound (23.33 mg/mL), followed by EFL (19.254 mg/mL) and PE (14.722 mg/mL). The highest content of flavonoids was EF (5.726 µg/mL), followed by EFL (5.463 µg/mL) and ER (4.805 µg/mL). The antioxidant activity of EFL (87.62%) was similar to the positive control validating the potential of this species as a functional food and the development of standardized herbal formulation that can be used for preventing of degenerative diseases and cellular aging.

Keywords: *Ageratum conyzoides*, unconventional vegetable, functional food, nutraceutics, DPPH.

RESUMO

Compostos fenólicos, flavonoides e ação antioxidante de folhas, flores e raízes de mentrasto

Ageratum conyzoides (mentrasto) tem valor sócio-econômico-cultural inestimável sendo utilizada como hortaliça não convencional na tradicional culinária de Minas Gerais. Na medicina popular é usada como purgativa, febrífuga, antiemética, em cólicas, úlceras e problemas uterinos. O objetivo deste trabalho foi quantificar o teor de compostos fenólicos, flavonoides e atividade antioxidante em extratos etanólicos de mentrasto, como etapa para validação analítica e bioanalítica do uso como medicamento fitoterápico e/ou como alimento funcional. Assim, nos extratos etanólicos de folhas (EF), flores (EFL) e raízes (ER) de mentrasto fizeram-se análises dos teores de compostos fenólicos, flavonoides e atividade antioxidante (% AAT). O doseamento de compostos fenólicos, em EF, EFL e ER, foi realizado pelo método de Folin-Ciocalteu. Na determinação do teor de flavonoides, nos extratos, utilizou-se vanilina em meio ácido e na avaliação antioxidante, a metodologia do sequestro do radical 2,2-difenil-1-picrilhidrazil (DPPH). O delineamento experimental foi em blocos casualizados; todas as análises foram feitas com quatro repetições, os dados submetidos à análise de variância e teste de média a 1% de significância. ER teve maior concentração de compostos fenólicos (23,333 mg/mL), seguido de EFL (19,254 mg/mL) e EF (14,722 mg/mL). O teor mais elevado de flavonoides foi em EF (5,726 µg/mL), seguido de EFL (5,463 µg/mL) e ER (4,805 µg/mL). A atividade antioxidante de EFL (87,62%) foi semelhante ao controle positivo indicando a potencial ação desta espécie como alimento funcional e o desenvolvimento de formulação fitoterápica padronizada que possa ser utilizada na prevenção de doenças degenerativas e no combate ao envelhecimento celular.

Palavras-chave: *Ageratum conyzoides*, hortaliça não convencional, alimentos funcionais, nutracêuticos, DPPH.

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Ageratum conyzoides (mentrasto or white-weed) belongs to the tribe Eupatoriae, family Asteraceae whose vast majority of plants is herbaceous. The genre *Ageratum* consists of about 30 species (Okunade, 2002).

In Brazil, mentrasto is also known as catinga-de-bode, catinga-de-Barão, erva-de-São-João, Maria-preta, erva-de-São-José, picão-roxo, erva-de-Santa-Lúcia, camará-opela, agerato, camará-apeba, camará-Iapó, camará-Japé, erva-de-Santa-Maria, macela-de-São-João, macela-francesa, matruço (Ming, 1999); whereas overseas, it is known as billy white-weed, white-weed (EUA, Australia), Rumput tahi ayam (Malaysia), Aru Batu (Indonesia),

mexican ageratum (Mexico), Z'herbe á femme (**Trinidad and Tobago) (**Shirwaikar *et al*.**, 2003; Tropical Plant Database, 2012).**

White-weed is found in tropical and subtropical regions, that means, has pantropical distribution (Ming, 1999). The genre occurs from southeastern North America to Central America, having as center of origin Central America and the Caribbean. Socially, it has diverse uses, from ornamental, forage for goats, cattle and mules in Malaysia, to flavoring white clothes in Northeastern Brazil (Castro *et al.*, 2004). Some studies report the use of this species as a hedge and windbreak in organic cultivation (Maia & Oliveira, 2004).

This plant is characterized as upright, herbaceous, annual, 30-80 cm tall, with stems and leaves covered with white trichomes, opposite leaves, long petioles pubescent with glandular trichomes. Flowers arranged in terminal inflorescences (Okunade, 2002) with 30-50 rose-purple flowers arranged in a corymb, which are self-incompatible. The fruit is easily dispersed by anemochory. Seeds are positive photoblastic, the optimum temperature for germination ranges from 20-25°C. The plant shows great morphological variation and it seems highly adaptable to different ecological conditions, having high resistance in the field (Ming, 1999).

White-weed has high variability of secondary metabolites, including flavonoids (kaempferol, quercetin and glycosides), alkaloids, coumarins, essential oils and tannins (Ming, 1999). It also contains volatile organic compounds such as mono and sesquiterpenes and non-volatile, such as gallic acid, coumalic, protocateico, benzoic, synapic, p-hydroxybenzoic acid and coumaric acid (Nogueira *et al.* 2010).

Phenolic or polyphenolic compounds are one of the most abundant plant metabolites, with more than 8,000 phenolic structures, currently known, widely distributed in the plant kingdom and being used in food (Soobrattee *et al.*, 2005). Natural polyphenols can range from simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerized compounds (lignins, tannins). Phytochemical investigations of the extract of *A. conyozoides* showed high concentrations of phenolic compounds and flavonoids with potential use for protection against disorders associated with excess of free radicals and reactive oxygen species in biological systems (Verma *et al.*, 2013).

In white-weed, conyzorigum, chromene, precocene II and precocene I were chemically identified (Ming, 1999). These secondary compounds are formed under adverse conditions such as injury and UV radiation and they have antioxidant properties. They are found in plants, in the form of phenolic compounds (flavonoid, acids, alcohols, stilbenes, tocopherols, tocotrienols), carotenoids and ascorbic acid (Bernardes *et al*., 2006). Phenoxyl radicals (present in phenolic compounds), formed through oxidative pathways, have multiple defenses in plant, due to its ability to start the free radical chain reactions in the cell membrane (Sakihama *et al*., 2002).

Bioactivity of terpenic compounds was detected in different white-weed accessions, mainly precocenes which act as anti-juvenile hormone besides being responsible for the effects of insecticides, the most important biological activity of this species (Okunade, 2002). The essential oil of white-weed also protects stored grain against the action of fungi (Castro *et al.*, 2004). Predominant compounds of the essential oil of white-weed, mainly chromenes precocene I and precocene II, cause premature metamorphosis in several species of insects, leading to the formation of sterile adults (Castro *et al.*, 2004).

The extract of white-weed has anti-inflammatory, antipyretic and analgesic activities in mice and rats. The oil has antibacterial and antifungal activity (Okunade, 2002). Studies on the phytochemical investigation of the plant extract of white-weed show high concentration of phenolic compounds and flavonoids, for protection against disorders associated with excess of free radicals or reactive oxygen species (Verma *et al.*, 2013).

The use of white-weed in stews as an unconventional leafy vegetable is a culinary tradition of Minas Gerais, considered as a Brazilian intangible heritage. Almeida *et al*. (2002) performed the mineral characterization of white-weed seeking to validate its potential as unconventional vegetable and found the following mineral contents Na^{+} (24.0±0.3%), K^{+} (74.0±1.3%), Ca^{2+} (854±16%), Mg^{2+} (244±5%), Fe^{3+} (3.476±0.068%), Al^{3+} (11.68±0.21%), Mn^{2+} (0.233 ± 0.005%), Zn^{2+} (0.233±0.005%) and concluded that in a comparative analysis to other plant products, this plant shows significant Ca^{2+} and Mg^{2+} concentrations which can validate its use as functional food.

The daily consumption of antioxidants can produce effective protective action against oxidative damage which naturally occurs in the body (Degáspari & Waszczynskyj, 2004). Global increases in cellular level of reactive oxygen species results in oxidative stress, which can lead to cell death. Oxidative stress is related to chronic diseases like cancer, diabetes, neurodegenerative and cardiovascular diseases (Degáspari & Waszczynskyj, 2004).

Epidemiological studies show that a diet high in fruits, vegetables and other plant products may be associated with lower risk of premature death and mortality related to cardiovascular disease and some cancers.

Pharmacologically, phenolic compounds, especially polyphenols, are responsible for these effects. In studies on Ehrlich ascitic tumor cells, the antitumor activity of white-weed was proven, attributing this effect to the chemical composition of the plant, especially the flavonoids (Momesso *et al.*, 2009).

Although numerous pharmacological studies on white-weed can be found, currently, no herbal formulation with the species exists in the Brazilian market of medicines, and still no content of bioactive compounds in herbal extracts was standardized.

Another relevant aspect is that although this species is known and appreciated by indigenous communities, no extensive cultivation is performed;

no study on which plant organ higher content of bioactive compound can be found, which hinders its use as a functional food. Thus, due to reported factors and the cultural, social and economic importance of this plant, the aim of this study was to quantify the content of phenolic compounds, flavonoids and antioxidant activity in ethanol extracts of leaves, flowers and roots of white-weed, as a validation of analytical and bioanalytical methods, aiming the future use as herbal medicine and/or functional food.

MATERIAL AND METHODS

White-weed was collected in UFOP campus (Universidade Federal de Ouro Preto), Ouro Preto, Minas Gerais state, Brazil (20°23'28"S, 43°30'20"W), in the early vegetative propagation in November 2011. After the collection, taxonomic species identification and mounting plant specimens at the Herbário Professor José Badini (OUPR) were performed. The identification was made by Dr. VR Scalon and, the voucher specimen is deposited under number OUPR25894.

Fully expanded adult plants were collected with approximately two months of development. After the collection, the authors segregated the plant material in fully expanded entire adult leaves, complete roots, fully expanded, and young flowers (pre-flowering) which were weighed obtaining 19.558 g of roots, 20.306 g of flowers and 12.770 g of leaves. The extracts were made with ethanol PA (CROMOLINE), using 500 mL in leaves (EF), 300 mL in flowers (EFL) and 450 mL in roots (ER) using the method of successive extractions by remaceration until the exhaustion of the plant material. Then, the material was filtered in radiated funnel and analytical filter paper, followed by complete evaporation in a 37°C water bath, resulting in leaf (EF), flower (EFL) and root (ER) dry extracts of white-weed. The yield of dry extracts was calculated according to the initial fresh mass.

The determination of the phenolic compounds was adapted from Murthy, Singh *et al.* (2002). The authors weighed 10 mg of dry extracts of EF, EFL and ER treatments that were added to 1000 µL of Ethanol PA (CROMOLINE), which retreated 40 µL, resulting in 0.004 mg/µL of EF, EFL and ER, respectively, added to 1000 µL of Folin-Ciocalteu reagent (VETEC) (1:10) and 800 µL of 7.5% sodium carbonate. Then, the dry extracts were incubated at an average temperature of 23°C for 30 minutes, measuring the absorbance using a spectrophotometer at 760 nm. Standard curve was constructed with tannic acid (Sigma) (0.1 mg/mL) as reference substance. The contents of phenolic compounds in EF, EFL and ER were expressed in mg/mL of tannic acid.

The content of flavonoids was determined using a method adapted from Jayaprakasha *et al*. (2001). The authors weighed 4 mg of EF, EFL and ER, which were suspended in 4 mL of Ethanol PA. Then, 100 µL aliquots of this solution was added to 2 mL of 4% hydrochloric acid and 2 mL of 10% ethanolic vanillin, homogenized and stored away from light and heat at 21°C for 30 minutes. Then, the reading was performed, using a spectrophotometer at 460 nm. The standard curve was constructed using quercetin (Sigma) (10 mg/mL) under the same conditions described. The flavonoids contents were expressed in µg/mL of quercetin.

The antioxidant activity assay was performed using the method of capture radical 2.2-diphenyl-1-picrylhydrazyl (DPPH) using the extracts of leaves (FE), flowers (EFL) and roots (ER) of white-weed and their dilutions to 500 ppm, in four replications, in accordance with the assay performed by Oliveira *et al*. (2011). In dark environment, an aliquot of 0.1 mL of the extract was transferred to test tubes with 1.0 mL (0.1 mM) of methanolic solution of DPPH (SIGMA). The mixture, homogenized by vortexing for 1 minute, was kept at rest at room temperature for 15 minutes, protected from light. This procedure was repeated for the extracts diluted at a concentration of 500 ppm. Subsequently, the reading of the extracts has been made at 517 nm with a spectrophotometer, using methanol as blank. In the positive control assay, butylhydroxyanisole (BHA, Sigma) to 100 ppm was used. In the calculation of the consumption of DPPH radical the following equation was used: AAT% = [(AbsControle - AbsAmostra) / AbsControle] x 100, where AbsAmostra corresponds to the absorbance of DPPH solution containing the sample and AbsControle.

The experimental design was in randomized blocks, with four replications for each treatment and the results were subjected to the analysis of variance and to test at 1% significance (SAEG, 2012).

RESULTS AND DISCUSSION

The ethanolic dry extracts of leaves (EF), flowers (EFL) and roots (ER) retained the characteristic color of the liquid extract EF (dark green), FR (yellow-brown), EFL (green-brown) and the distinctive smell as fresh plant (nauseating, woody). The yield of dry extracts, calculated according to the initial fresh mass, were 2.40%; 1.51% and 0.44% in EF, EFL and ER,

Table 1. Mean values of phenolic compounds concentrations (mg/mL) and flavonoids (µg/mL) in extract of leaves (EF), flowers (EFL) and roots (ER) of white-weed (*Ageratum conyzoides*) [valores médios de teores de compostos fenólicos (mg/mL) e flavonoides (µg/mL) em extrato de folhas (EF), de flores (EFL) e de raízes (ER) de mentrasto (*Ageratum conyzoides*)]. Ouro Preto, UFOP, 2012.

Treatment	Phenolic compounds (mg/mL)	Flavonoids (µg/mL)
EF	14.722 c	5.726 a
EFL	19.254 b	5.463 ab
ER	23.333 a	4.805 b
CV (%)	5.58	5.20

Means followed by the same letter in the column were not significant, Tukey 1% (médias seguidas de uma mesma letra na coluna não foram significantes, Tukey 1%).

respectively.

For the determination of phenolic compounds, tannic acid used as standard ($\hat{y}$= 0.0342x – 0.156; r^2= 0.9987). The average concentration of phenolic compounds in the treatments (EF, EFL and ER) was 19.1033±3.7979 mg/mL of tannic acid. The average contents of phenolic compounds in EF, EFL and ER were, 14.7222 mg/mL; 19.2544 mg/mL and 23.333 mg/mL, respectively. ER showed the higher concentration followed by EFL and EF (Table 1).

It is important to point out that the contents of phenolic compounds, plant defense metabolites, were higher in roots (ER), and it may be related to the structures of lignification and resistance, which offer greater hardness, low tissue elasticity and consequent physical barrier to soil pathogens (Sakihama *et al.*, 2002). It is noteworthy that white-weed is considered an invasive species which is difficult to prevent, control and eradicate, competing for water, nutrients, light, affecting the germination and growth of other species. This property can be justified by the fact that white-weed can release alelo-chemical substances on crops grown, such as radish, mung bean and ryegrass (Guimarães *et al*., 2012). EFL shows significant concentration of phenolic compounds, which, possibly, is related, in flowers, to protection against herbivory of reproductive structures. As a vegetable, the leaves have lower content of FC, which gives better flavor in stews.

For the determination of flavonoids, quercetin was used as standard ($\hat{y}$= 0.0002x + 0.0563; r^2= 0.9946). The average concentration of flavonoids in the treatments was 5.3316 ± 0.4761 mg/mL expressed in quecertina (Table 1). The highest content of flavonoids was in EF (5.7263 µg/mL), followed by EFL (5.4632 µg/mL) and ER (4.8053 µg/mL) (Table1). It is important to highlight that flavonoids are compounds biologically related to protection against UV rays, attraction of pollinators and defense against herbivory. The species was collected at the beginning of flowering (2 months of cultivation), in full vegetative growth and as indication of ethnobotanical surveys, when its use in cooking is more effective. The authors expected, therefore, according to the life cycle of the species that they would translocate compounds related to defense and pollinator attraction (purplish color), to the structures responsible for the formation of seeds and resultant dispersion, which was in accordance with the age of the plant studied and the results found, once the content in leaf is higher than the content in flower.

Table 2. Mean values of consumption of DPPH (%) in extract of leaves (EF and EF500) of flowers (EFL and EFL500) and of roots (ER and ER500) of white-weed (*Ageratum conyzoides*) and positive control butylhydroxyanisole (BHA) (100 ppm) [valores médios de consumo de DPPH (%) nos extratos de folhas (EF e EF500), de flores (EFL e EFL500) e de raízes (ER e ER500) de mentrasto (*Ageratum conyzoides*) e controle positivo butilhidroxianisol (BHA) (100ppm)]. Belo Horizonte, UFMG, 2012.

Treatment	Consumtion of DPPH (%)
EF	83.15 ab
EF500	75.58 c
EFL	87.62 a
EFL500	78.69 bc
ER	41.91 d
ER 500	34.67 e
BHA	86.94 a
CV (%)	2.83

Means followed by the same letter in the column were not significant, Tukey 1% (médias seguidas de uma mesma letra na coluna não foram significantes, Tukey 1%).

The results of antioxidant activity are shown in Table 2. Comparing the extracts of white-weed to the standard of BHA used, the antioxidant activity of leaf extracts (EF) and flowers (EFL) of white-weed was verified, highlighting in this case, the flower extract whose activity exceeded the standard used. The antioxidant activity test showed that the fractions of white-weed have the ability to capture free radicals and neutralize them. The extracts obtained from the roots also showed antioxidant potential, however, lower when compared to the other samples (Table 2). It is worth highlighting the persistence of high level of antioxidant activity of the extracts evaluated, even being subjected to dilution. To support this fact, is the color change observed during the procedure, that is, violet to yellowish, which confirmed the capture of DPPH and resulted in a decrease of absorbance values obtained.

Among the metabolites responsible for the antioxidant activity, the phenolic compounds stand out, whose redox potential plays an important role in adsorbing and neutralizing free radicals (Lima *et al*., 2006). Phenolic compounds, plant defense metabolites, showed higher concentration in roots, which can be related to the presence of lignin content, providing, hardness, lignification of tissues and subsequent physical strength, promoting root growth and expansion. Lignin acts hindering herbivory. In literature, white-weed has allelopathic properties of both essential oil and aqueous extract (Okunade, 2002). The flowers also show significant concentration of phenolic compounds, which, possibly, is related to protection against herbivory of reproductive structures.

Pharmacologically, the highest content in leaves (EF) of white-weed validates the popular use and permeates future studies aiming to produce herbal medicines. Momesso *et al.* (2009) point out studies which were carried out determining proven antitumor activity of the plant, attributing these effects to the concentration of flavonoids. Furthermore, Sakihama *et al.* (2002) state that flavonoids comprise a source of potent natural antioxidants. Flavonoids possess ideal structure for free radical scavenging activities. The antioxidant activity of flavonoids depends on their structure.

Comparing the data for the

determination of this plant antioxidants to similar studies which contemplated *Baccharis dracunculifolia* (alecrim-do-mato), *Taraxacum officinale* (dente-de-leão) and *Bidens segetum* species (picão-do-mato), it is possible to observe a positive pattern in plant species of Asteraceae, justified by its composition rich in phenolics, especially flavonoids and tannins (Momesso *et al*., 2009).

The data obtained showed that the ethanol extracts of leaves and flowers (aerial parts) have lower levels of phenolic compounds, however, flower extract has higher antioxidant activity, allowing suggesting the use of flowers in cooking, as well as the leaves, validating its use as a functional food, either in salad or stew.

One of the most complete definitions regarding the functional food, describes them as natural foods or ingredients that benefit one or more bodily functions beyond basic nutrition contributing through their bioactive compounds and pharmacological activities to improve the health and welfare and/or reduce the risk of diseases (Carvalho *et al.*, 2006; Hasler 2002; Neves *et al.*, 2009). This beneficial effect on human health occurs mostly when they are eaten as part of a usual diet (Cardoso & Oliveira, 2008), supported by the traditional use as found in the reports on the popular use of white-weed in cooking.

The high contents of phenolic compounds in roots associated with intermediate levels of flavonoids suggest the development of antiparasitic herbal pharmaceutical formulation or natural defensive of extract of white-weed roots.

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