Effects of extraction methods of phenolic compounds from *Xanthium strumarium* L. and their antioxidant activity

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RESUMO: Foram avaliados os efeitos de métodos de extração e de solventes sobre o rendimento de extrato, conteúdo de fenólicos totais, atividade antioxidante, e composição dos compostos fenólicos de *Xanthium strumarium* L. A atividade antioxidante foi determinada pelo método do radical 2,2-difenil-1-picrilhidrazila (DPPH), e a composição dos compostos fenólicos foi determinada por cromatografia líquida de alta eficiência e por espectrometria de massas com ionização por *electrospray*. Todos os resultados foram afetados pelo método de extração e, principalmente pelo solvente utilizado, sendo que os melhores resultados foram obtidos com o solvente metanol. Os extratos metanólico e etanólico apresentaram forte ação antioxidante e os principais compostos fenólicos encontrados nos extratos foram os ácidos ferúlico e clorogênico.

Palavras-chave: *Xanthium strumarium*, carrapicho, atividade antioxidante, compostos fenólicos, IAA

ABSTRACT: Efeito de métodos de extração de compostos fenólicos de *Xanthium strumarium L.* e suas atividades antioxidantes. The effect of extraction methods and solvents on overall yield, total phenolic content, antioxidant activity, and the composition of the phenolic compounds in *Xanthium strumarium* extracts were studied. The antioxidant activity was determined by using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), and the composition of the phenolic compounds was determined by HPLC-DAD and LC/MS. All results were affected by the extraction method, especially by the solvent used, and the best results were obtained with the methanol extract. The methanolic and ethanolic extracts exhibited strong antioxidant activity, and the chlorogenic and ferulic acids were the most abundant phenolic compounds in the extracts.

Keywords: *Xanthium strumarium*, common cocklebur, antioxidant activity, phenolic compounds, AAI.

INTRODUCTION

Xanthium species have been used as traditional herbal medicines for centuries in Oriental countries. In Brazil, it is found in popular herbal stalls. The whole plant has been used to treat bacterial infections, diabetes, skin pruritus, inflammatory diseases like rhinitis and rheumatoid arthritis, and cytotoxicity and antitumor activity (Kamboj & Saluja, 2010; Kumar & Rajkapoor, 2010; Patil et al., 2012; Aranjani et al., 2013). Despite the medicinal use, some investigations have reported that *Xanthium strumarium* induced intoxication and could be lethal to cattle (Colodel et al., 2000), sheep (Loretti et al., 1999), pigs (Stuart et al., 1981; Masvingwe & Mavenyengwa, 1998), and humans (Turgut et al., 2005). The results showed that the consumption of the fruits (burrs) induced hepatic necrosis, as well as myocardial injury in humans. The toxic substance of *X. strumarium* was isolated and identified as carboxyatractyloside (CAT) (Cole et al., 1980), a highly selective inhibitor of oxidative phosphorylation (Scott et al., 1993). The presence of CAT in the adult leaves, cotyledonary stages, seeds (inside the burr), and the shell of the burr was investigated via ESI-MS/MS, and the CAT was found in the extracts from the seed and plants in the cotyledonary stage, but not in the adult leaves or shell of burr, so the medicinal

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use of *X. strumarium* should be restricted to the adult leaves (Scherer et al., 2009).

The interest in natural sources of antioxidant molecules for use in the food, beverage, and cosmetic industries has resulted in a large field of research in recent years. It is well known that natural antioxidants extracted from herbs and spices have high antioxidant activity and are used in many food applications. Among these substances, phenolic compounds, which are widely distributed in plants, have the ability to sc avenge free radicals by single electron transfer (Hirano et al., 2001).

Several investigations have reported the biological properties of *Xanthium strumarium* L., such as its anti-ulcerogenic (Favier et al., 2005), larvicidal and repellent (Singh et al. 2009), anti-helmintic (Sharma et al., 2003), anti-inflammatory (Huang et al., 2011), diuretic (Nieves et al., 1999), antioxidant (Ishwarya & Singh, 2010) antimicrobial (Scherer et al., 2009), and antilipidemic actions (Sridharamurthy, et al. 2011). However, there are no reports about antioxidant activity of Brazilian plants. Therefore, the aim of this work was to investigate the antioxidant activity of several extracts of *X. strumarium* leaves from Brazil, as well as the related phenolic compounds.

MATERIAL AND METHOD

Material

The solvents used were methanol, ethanol, ethyl acetate, chloroform, and dichloromethane Ecibra (Brazil), and methanol Mallinckrodt (USA) HPLC grade. Free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma (USA). Folin-ciocalteau reagent was obtained from QEEL (Brazil), and sodium carbonate was from Nuclear (Brazil). The synthetic antioxidant butylated hydroxyanisole (BHA) and the phenolic compounds caffeic acid, chlorogenic acid, ferulic acid, gallic acid, quercetin, rutin, protocatechuic acid (3,4 dihydroxybenzoic acid), and *trans*-cinnamic acid were purchased from Sigma (USA).

Plant material

The *X. strumarium* used in this work was cultivated in the experimental farm of the Faculty of Agricultural Engineering (FEAGRI) of the State University of Campinas (UNICAMP, Campinas, São Paulo, Brazil). A voucher specimen was deposited at the State University of Campinas Herbarium as n° UEC 134865. The leaves, harvested in April, were separated, dried in a tray dryer with air circulation at 45°C (Marconi, model MA035, Brazil), packed in dark plastic bags, and stored in a freezer at −20°C until the extraction.

Extractions

Before the extractions, the leaves were triturated in a food processor, and particles with sizes from 24 - 48 mesh were classified using an electromagnetic vibrator for 10 minutes (Bertel, Model 1868, Brazil). The extractions were carried out using 3 different extraction methods and 4 different solvents in triplicate. The methods were static maceration (1), dynamic maceration (2), and Soxhlet extractor (3), and the solvents were 80% ethanol (A), 80% methanol (B), ethyl acetate (C), and chloroform/ dichloromethane (1:1) (D). Maceration was carried out with 20 g plus 100 mL of the different solvents. After 7 days with periodic agitation, the extracts were filtered through paper, and the residue was re-extracted with 100 mL of the respective solvents with 10 min stirring. Both fractions were then joined and evaporated to dryness at 38°C under vacuum. Dynamic maceration was carried out with 20 g plus 100 mL of the different solvents. After 3h of shaking, the extracts were filtered through paper, and the residue was extracted again with 100 mL of the respective solvents for 1h. Both fractions were then blended and evaporated to dryness at 38°C in a vacuum. The Soxhlet method was carried out in a Soxhlet apparatus with 15 g for 5 h of extraction with the solvents A, B, and C. The extracts were dried at 38°C in a vacuum. All extracts were stored in a freezer at −20°C until analyzed.

Total phenolics content (TPC)

The total phenolics content was determined using the Folin-Ciocalteau reaction. An aliquot of 0.5 mL of a methanolic solution of dry extracts (1.5 mg mL^{-1}) was added to 2.5 mL of Folin-Ciocalteau reagent diluted with water (1/11). After 5 minutes 2.0 mL of 7.5% sodium carbonate was added and stirred vigorously in a vortex mixer (Biomixer, VTX-2500, Brazil). The mixture was maintained for 2 h in the dark at room temperature (22 ± 2 ºC). The absorbance at 740 nm was measured and converted to the phenolics content according to a calibration curve made with gallic acid (2.0, 1.0, 0.5, 0.25, 0.125 mg mL^{-1}).

Antioxidant activity

The antioxidant activity of the extracts and standards was determined from the radical scavenging activity determined using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), according to Scherer and Godoy (2009). Aliquots of 0.1 mL of methanol solutions of samples or standards in different concentrations were added to 3.9 mL of a methanol solution of DPPH (0.2 mM). The blank sample consisted of 0.1 mL of methanol added to 3.9 mL of DPPH solution. The tests were carried out in triplicate. After a 90-min incubation period at room temperature in the dark, the absorbance was measured at 517 nm.

The radical scavenging activity was calculated as follows: I% = [(Abs0 - Abs1)/Abs0] x 100, where Abs0 was the absorbance of the blank and Abs1 was the absorbance in the presence of the test compound at different concentrations. The IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve in the linear range by plotting the extract concentration vs the corresponding scavenging effect. The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated as follows as: AAI = final concentration of DPPH (µg/mL) / IC_{50} (µg/mL). The assays were carried out in triplicate, and all the samples and standard solutions, as well as the DPPH solutions, were prepared daily.

Chromatographic analyses

Analyses were performed using an HP 1100 series liquid chromatograph (Agilent, USA) equipped with degasser, quaternary pump, autosampler and a UV-visible detector diode array (DAD) set at 280 and 320 nm. The separation was performed using a VYDAC™ RP-C18 column (5 µm particle size, 250 x 4.6 mm id., 25°C) with a gradient elution procedure using a flow of 0.7 mL min^{-1}. The mobile phase consisted of water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B). The gradient elution started with 85% A and 15% B, rising to 50% A and 50% B after 10 minutes, and 20% A and 80% B in 25 minutes. The column equilibrium time before each new injection was 10 minutes in the initial condition. The compounds were identified by comparison of retention time and spectrum with those of the standard solution. Quantification was performed by external curve with 7 points. The following validation parameters were evaluated: selectivity, linearity range, precision and limits of detection, and quantification. The selectivity was verified from the diode array spectra. The repeatability was evaluated with 10 consecutive tests, while the intermediate precision was assessed with three tests on three different days. The limits of detection and quantification were determined by the signal noise, 3 and 5 times respectively.

LC-ESI-MS-MS-TOF analysis

To confirm the identity of phenolic compounds, samples were analyzed by direct infusion (FIA) in a mass spectrometer Q-TOF microTM (Waters/Micromass, USA) using a liquid chromatograph Alliance 2695 (Waters, USA) for injection. The mobile phase consisted of water with 0.8% NH_4OH and methanol (1/1) in isocratic elution procedure with a flow of 0.1 mL min^{-1}. The mass spectra were obtained with electrospray ionization (ESI) in negative mode range from 100 to 400 m/z. The phenolic compounds were identified by comparing the MS/MS mass spectrum with standard solutions.

Statistical analysis

The data obtained were analyzed using ANOVA/Tukey ($p < 0.05$). The statistical package used was Statistica™ 6.0 data analysis software by Statsoft, Inc, USA.

RESULTS AND DISCUSSION

Table 1 shows the extraction yields and total content of phenolic compounds (TPC). The efficiency of the extraction yield ranged from 4.6% to 26.4% (w/w), depending on the extraction method and solvent used. The Soxhlet method showed the highest yield with the solvent methanol, followed by ethanol. The high yield of Soxhlet method can be explained by the temperature, which increased the strength of solvation. Kumar and Rajkapoor (2010) evaluated the extraction yield of whole plant by petroleum ether (60°-80°C) using Soxhlet apparatus, they reported an extraction yield of about 9%. This value was lower than the values obtained with polar solvents in the present work, but near to the value obtained with the ethyl acetate. The solvent ethyl acetate and the solvent chloroform/dichloromethane had low incomes, and no difference was observed between them. These results agree with previous studies (Moure et al., 2000). The content of total phenolic compounds, expressed as gallic acid equivalents, was affected by the method of extraction and the solvent used (Table 1), and the best results obtained were, in descending order, with the solvent methanol, ethanol, ethyl acetate and mixture. The maceration with methanol showed the highest TPC in the extract.

TABLE 1. Yield and total phenolic content (TPC) of the *Xanthium strumarium* extracts.

Extracts	Yield (%)	TPC (mg g^{-1} extract)
1A	12.3 ± 0.5^{d}	64.51 ± 1.0^{d}
2A	14.1 ± 1.0^{cd}	70.07 ± 1.6^{c}
3A	19.3 ± 0.8^{b}	69.38 ± 1.3^{cd}
1B	14.3 ± 0.3^{cd}	93.68 ± 2.1^{a}
2B	14.9 ± 1.3^{c}	78.23 ± 0.9^{b}
3B	26.4 ± 1.3^{a}	81.35 ± 1.3^{b}
1C	6.5 ± 0.4^{ef}	21.98 ± 3.6^{f}
2C	4.6 ± 0.6^{f}	27.19 ± 1.0^{e}
3C	6.9 ± 0.3^{e}	23.19 ± 0.3^{ef}
1D	6.2 ± 0.4^{ef}	13.30 ± 0.8^{g}
2D	5.3 ± 0.2^{ef}	18.85 ± 2.4^{f}

1: static maceration; 2: dynamic maceration; 3: Soxhlet; A: 80% ethanol; B: 80% methanol; C: ethyl acetate; D: chloroform/dichloromethane (1:1). The results are the mean of triplicate. Values with different superscripts in the same column were significantly different ($p < 0.05$). TPC: total phenolic compound.

Table 2 shows the results of the Antioxidant activity index (AAI) for the extracts and pure compounds. For the IC_{50}, it is very important that the determination should be done in a linear range for each compound. Therefore, a calibration curve was performed for all compounds tested, and good linear ranges were observed (0.9809-1.0000). The linear range and stability of DPPH solutions were evaluated in a previous study (Scherer & Godoy, 2009). The results showed no difference in absorbance between 0 and 90 min for any of the concentrations tested, and good linear ranges were observed.

The antioxidant activity of extracts of *Xanthium strumarium* was affected by the extraction method and the solvent used. According to Scherer and Godoy (2009), AAI values below 0.5 indicate low antioxidant activity, values between 0.5 and 1.0 indicate moderate activity, values between 1.0 and 2.0 indicate a strong activity, and AAI values above 2.0 indicate very strong antioxidant activity. Considering this classification, the extracts obtained with nonpolar solvents ethyl acetate and the mixture (chloroform/dichloromethane) had IC_{50} values very high and, consequently, low AAI values, indicating weak antioxidant activity. On the other hand, both polar extracts showed strong antioxidant activity (Table 2).

Gallic acid had the highest value of AAI, followed by protochatechuic acid and quercetin. There was no significant difference between chlorogenic acid, caffeic acid, and BHA, but they all showed AAI values higher than ferulic acid and rutin (Table 2). Trans-cinnamic acid showed no ability to reduce DPPH, even at the highest concentration tested (200 μg mL^{-1} of final concentration).

Phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds (Bravo, 1998). The structure of phenolic compounds is a key determinant of their radical scavenging and metal chelating activities, and this is referred to as structure-activity relationships (Balasundram et al., 2006). The antioxidant activity of phenolic acids increases with increasing degree of hydroxylation, as is the case with gallic acid (trihydroxylated) and protochatechuic acid (dihydroxylated), which show high AAI values. The replacement of the hydroxyl group on the aromatic ring with a methoxyl group, as in the case of caffeic acid to ferulic acid, reduced the value of AAI (Table 2), according to Rice-Evans et al. (1996), who reported that the substitution of hydroxyl groups by methoxyl reduces antioxidant activity. This can be explained by reduced ability to donate a hydrogen atom of the molecule. The absence of a hydroxyl group on the aromatic ring of the trans-cinnamic acid explains its failure to reduce

TABLE 2. Antioxidant activity index (AAI) of *Xanthium strumarium* extracts.

Extract/pure compound	*IC_{50} (mean ± SD)	AAI (mean ± SD)
1A	47.83 ± 1.40	1.61 ± 0.05^{g}
2A	53.01 ± 1.20	1.45 ± 0.03^{g}
3A	53.34 ± 1.52	1.44 ± 0.04^{g}
1B	44.94 ± 1.06	1.71 ± 0.04^{f}
2B	45.05 ± 1.15	1.70 ± 0.03^{f}
3B	43.53 ± 1.61	1.77 ± 0.07^{f}
1C	346.35 ± 16.50	0.22 ± 0.01^{h}
2C	369.83 ± 13.58	0.21 ± 0.01^{hi}
3C	423.97 ± 22.27	0.18 ± 0.01^{hi}
1D	657.10 ± 24.01	0.12 ± 0.00^{i}
2D	674.61 ± 28.57	0.11 ± 0.00^{i}
Gallic acid	2.83 ± 0.07	27.1 ± 0.68^{a}
Protochatechuic acid	3.82 ± 0.15	20.2 ± 0.77^{b}
Quercetin	4.88 ± 0.56	15.9 ± 1.76^{c}
Chlorogenic acid	7.44 ± 0.12	10.3 ± 0.17^{d}
Caffeic acid	8.21 ± 0.31	9.4 ± 0.35^{d}
BHA	8.23 ± 0.41	9.3 ± 0.46^{d}
Rutin	12.09 ± 0.70	6.4 ± 0.37^{e}
Ferulic acid	14.45 ± 0.66	5.3 ± 0.25^{e}
trans-cinnamic acid	NA	NA

1: static maceration; 2: dynamic maceration; 3: Soxhlet; A: 80% ethanol; B: 80% methanol; C: ethyl acetate; D: chloroform/dichloromethane (1:1). Values with different superscripts in the same column were significantly different ($p < 0.05$). *Inhibitory concentration of 50% of the DPPH radicals (μg mL^{-1}). NA: No ability to reduce DPPH

the DPPH. The structure-activity relationship of flavonoids is usually more complicated than that of hydroxybenzoic and hydroxycinnamic acids due to the relative complexity of the flavonoid molecules. The degree of hydroxylation, as well as the position of the hydroxyl groups, increases the scavenging ability of free radicals of flavonoids (van Acker et al., 1996). For this reason, quercetin has a value greater than the AAI rutin (Table 2), in which the hydroxyl group was replaced by rutinose.

The presence of trans-cinnamic acid and the phenolic compounds caffeic acid, chlorogenic acid, ferulic acid, gallic acid, quercetin, rutin, and protocatechuic acid was measured by HPLC-DAD. The analysis of the extracts revealed the presence of three compounds: chlorogenic acid, ferulic acid, and trans-cinnamic acid. Identification of these compounds was confirmed by ESI-MS/MS analysis. The electrospray ionization in negative mode is a sensitive and selective method for the identification of polar organic compounds with acidic sites, such as phenolic compounds. Thus, the deprotonated forms $[M-H]^-$ of the compounds of interest were selected, and their fragments were monitored. The results showed that chlorogenic acid has molecular ion 353.0858 m/z and four main fragments: 191.05, 85.03, 93.03 and 127.04. Ferulic acid, with molecular ion 193.0512 m/z, showed three fragments: 178.04, 134.04 and 149.07. And trans-cinnamic acid showed the molecular ion 147.0457 m/z and two fragments: 77.04 and 103.05.

Table 3 shows the results of the HPLC-DAD for chlorogenic acid, ferulic acid, and trans-cinnamic acid in extracts of *Xanthium strumarium*. The methanol extracts showed the highest values for chlorogenic and ferulic acids. The solvents ethyl acetate and chloroform/dichloromethane (1:1) showed less ability to extract the phenolic compounds, with the lowest values for all compounds. This can be explained by lower solubility of phenolic compounds in solvents of low polarity.

As previously mentioned, the methanol extracts showed higher contents of total phenolics and the highest values of AAI, followed by ethanol extracts, and the lowest values were found with the solvents ethyl acetate and chloroform/dichloromethane (1:1). Some studies have reported a high correlation between the antioxidant activity of plant extracts with the content of phenolic compounds (Sun & Ho, 2005; Singh et al., 2007). In this study, the results showed a high degree of correlation ($r^2 = 0.97$) between total phenolic content and the AAI. In addition, we found a high correlation between AAI and chlorogenic acid ($r^2 = 0.97$) and ferulic acid ($r^2 = 0.96$), while the trans-cinnamic acid showed a lower degree of correlation ($r^2 = 0.90$). Since trans-cinnamic acid has no ability to reduce DPPH, the ferulic and chlorogenic acids have an important contribution to the antioxidant activity of *Xanthium strumarium*.

TABLE 3. HPLC-DAD analyses of chlorogenic, ferulic and *trans*-cinnamic acids in the *Xanthium strumarium* extracts.

Validation parameters	Chlorogenic acid	Ferulic acid	*trans*-cinnamic acid
Linearity (r^2)	0.9992	0.9999	0.9980
Repeatability (RSD %)	1.53	1.07	1.03
Intermediate precision (RSD %)	2.10	1.52	1.49
Limit of detection ($\mu g\ mL^{-1}$)	0.07	0.03	0.07
Limit of quantification ($\mu g\ mL^{-1}$)	0.12	0.06	0.13
Extract (mg g^{-1} of dry extract)			
1A	18.18 ± 0.72	23.12 ± 0.89	75.03 ± 0.27
2A	17.13 ± 0.40	22.92 ± 0.62	76.31 ± 0.59
3A	16.23 ± 0.35	26.55 ± 0.22	80.80 ± 0.62
1B	28.28 ± 0.42	39.01 ± 0.41	67.13 ± 0.71
2B	24.97 ± 0.75	30.47 ± 0.58	71.45 ± 0.31
3B	23.95 ± 1.09	41.85 ± 0.69	52.48 ± 0.04
1C	0.12 ± 0.00	0.74 ± 0.06	29.33 ± 0.18
2C	0.03 ± 0.00	0.12 ± 0.01	24.11 ± 0.27
3C	0.08 ± 0.01	0.26 ± 0.01	36.45 ± 0.34
1D	0.05 ± 0.00	0.11 ± 0.00	23.25 ± 0.36
2D	0.03 ± 0.00	0.06 ± 0.00	22.53 ± 0.07

RSD: relative standard deviation; 1: static maceration; 2: dynamic maceration; 3: Soxhlet; A: 80% ethanol; B: 80% methanol; C: ethyl acetate; D: chloroform/dichloromethane (1:1).

CONCLUSION

The yield, total phenolic content, and antioxidant activity were affected by the extraction method, and especially by the solvent used, and the best results were obtained with methanol and ethanol. There was a strong antioxidant activity in extracts of methanol and ethanol, but poor antioxidant activity for ethyl acetate and chloroform/ dichloromethane (1:1) extracts. A high degree of correlation between the total phenolic content and antioxidant activity was found. The phenolic compounds chlorogenic acid and ferulic acid greatly contribute to the antioxidant activity of extracts of *Xanthium strumarium*, which can be an alternative antioxidant for use in food or animal feed.

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