

***Xanthium strumarium* L. antimicrobial activity and carboxyatractyloside analysis through electrospray ionization mass spectrometry**

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ABSTRACT: The aim of this work was to evaluate the antimicrobial activity of *Xanthium strumarium* L. leaf extracts against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Clostridium perfringens*, as well as to investigate the presence of the toxic compound carboxyatractyloside in different plant parts. *S. aureus* and *C. perfringens* were more sensitive to non-polar than to polar fractions, and there was no difference between extracts for the remaining bacteria. All extracts had strong antimicrobial activity against the evaluated microorganisms. Carboxyatractyloside was found in cotyledons and seeds but not in adult leaves and burrs. Thus, only *Xanthium strumarium* leaves in adult stage can be used for medicinal purposes.

Key words: cocklebur, antimicrobial activity, medicinal plants

RESUMO: Atividade antimicrobiana e análise de carboxiatractilósido por espectrometria de massas com ionização por electrospray de *Xanthium strumarium* L. O objetivo do presente trabalho foi avaliar a atividade antimicrobiana de extratos de folhas de *Xanthium strumarium* L. sobre os microrganismos *Staphylococcus aureus*, *Escherichia coli*, *Salmonella thyphimurium*, *Pseudomonas aeruginosa* e *Clostridium perfringens*, bem como verificar a presença do composto tóxico carboxiatractilósido em diferentes partes da planta. As bactérias *S. aureus* e *C. perfringens* foram mais sensíveis às frações não polares do que as polares, sendo que para as outras bactérias não foi verificada diferença entre os extratos. Todos os extratos apresentaram uma forte ação antimicrobiana sobre os microrganismos avaliados. O carboxiatractilósido foi encontrado nos cotilédones e nas sementes da planta, entretanto, não foi encontrado nas folhas em estágio adulto e na carapaça espinhosa que envolve a semente. Portanto, somente as folhas de *Xanthium strumarium* na fase adulta podem ser utilizadas para o uso medicinal.

Palavras-chave: carrapicho, atividade antimicrobiana, plantas medicinais

INTRODUCTION

Cocklebur (*Xanthium strumarium* L.) is an herbaceous annual plant of worldwide distribution. The plant is erect, up to 2.5 m tall, and presents blotched purple stems. Its leaves are dark green on the upper surface, similar in shape to grape leaves, 15 cm in diameter and roughly textured with minute bristles. The flowers are inconspicuous, both male and female, occurring in leaf axils towards the end of the branches.

In addition, the flowers develop into hard woody burrs (fruits), 1.2 cm to 2 cm long, with numerous hooked spines. *X. strumarium* is extremely competitive with other crops. It has long been considered one of the worst weeds in soybean plantations (Bozsa & Oliver, 1993).

Some biological properties of *Xanthium strumarium* L. have been reported, such as anti-ulcerogenic (Favier et al., 2005), antitrypanosomal

(Talakal et al., 1995), anthelmintic (Sharma et al., 2003), anti-inflammatory (Kim et al., 2005; Yadava & Jharbade, 2007), diuretic (Nieves et al., 1999), antileishmanial, antifungal (Lavault et al., 2005) and hypoglycemic actions (Hsu et al., 2000), besides inhibition of cultured human tumor cell proliferation (Kim et al., 2003) and a significant depressant action on the central nervous system (Mandal et al., 2001).

Previous studies have reported that *X. strumarium* induces intoxication and can be lethal to cattle (Colodel et al., 2000), sheep (Loretti et al., 1999), pigs (Stuart et al., 1981) and humans (Turgut et al., 2005). Also, the consumption of fruits (burrs) and cotyledonary-stage leaves (two-leaf stage) leads to hepatic necrosis and myocardial injury in humans. The toxic principle in *X. strumarium* poison was isolated and identified as carboxyatractyloside (CAT) (Cole et al., 1980), a highly selective inhibitor of oxidative phosphorylation (Scott et al., 1993).

The chemical composition of *X. strumarium* includes phenolic compounds like chlorogenic and ferulic acids, thiazinediones (Han et al., 2006; Qin et al., 2006), triterpenoid saponin (Yadava & Jharbade, 2007), CAT (Cole et al., 1980), xanthanolide sesquiterpene lactones (8-epi-xanthatin and 8-epi-xanthatin epoxide) (Kim et al., 2003), several xanthanolides (Riscala et al., 1994), beta-sitosterol (Bisht et al., 1977), strumasterol (C-24 epimer of stigmasterol) (Bisht et al., 1978), monoterpene and sesquiterpene hydrocarbons (Taher et al., 1985), caffeic acid, 1,3,5-tri-O-caffeoyl quinic acid, 1,5-di-O-caffeoyl quinic acid (Sheu et al., 2003).

However, there are no reports about the antimicrobial activities of *X. strumarium* extracts against human pathogenic microorganisms such as *Escherichia coli* and about the presence of the toxic compound in different plant parts. Therefore, the aim of this work was to investigate the antimicrobial activities of several extracts from *X. strumarium* adult leaves against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Clostridium perfringens*, as well as to verify the presence of CAT in different plant parts.

MATERIAL AND METHOD

Reagents and standards

Chromatographic grade methanol (Tedia, Fairfield, OH, USA), ammonium hydroxide (Merck, Darmstadt, Germany), nutrient agar (Merck, Germany), dimethyl sulfoxide (DMSO) (Sigma, USA), triphenyl tetrazolium chloride (TTC) (Merck, Germany), culture medium (Mueller-Hinton 2.1%, Merck, Germany) were used. The antibiotics amoxicillin, enrofloxacin, erythromycin, lincomycin, tetracycline, oxytetracycline, penicillin G, spiramycin, sulfadiazine

and sulfanilamide were purchased from Sigma (USA). CAT was from Calbiochem (Darmstadt, Germany).

Plant material

Xanthium strumarium (common name: cocklebur, Family: *Asteraceae*) used in this work was cultivated in the experimental field of the School of Agricultural Engineering (FEAGRI), State University of Campinas (UNICAMP, Campinas, São Paulo State, Brazil). A voucher specimen was deposited at the State University of Campinas Herbarium under the number 134865 and identified by Dr. Washington M. F. Neto (curator). The leaves were separated, dried in a tray drier with air circulation at 45°C (Marconi, model 035, Piracicaba, São Paulo State, Brazil), packed in dark plastic bags and stored in a domestic freezer at -20 °C until extracted. Before extraction, the material was triturated in a domestic food processor (Wallita, model Master, São Paulo, SP) and the particles from 24 - 48 mesh selected using a magnetic agitator (Bertel, Model 1868, Caieiras, São Paulo State, Brazil).

Extracts

Extracts were obtained using 3 different extraction methods and 4 different solvents, in triplicate. The employed methods were maceration (1), dynamic maceration (2) and soxhlet (3). Solvents were 80% ethanol (A), 80% methanol (B), ethyl acetate (C) and dichloromethane/chloroform (1:1) (D). Maceration was done with 20 g plus 100 mL of the different solvents, resulting in the extracts 1A, 1B, 1C and 1D. After 7 days under periodic agitation, the extracts were filtered through paper filter and the residue again extracted with 100 mL of the respective solvents for 10 min under agitation. Both fractions were then blended and evaporated to dryness at 38°C under vacuum. Dynamic maceration was carried out with 20 g plus 100 mL of the different solvents, yielding the extracts 2A, 2B, 2C and 2D. After 3 h agitation, the extracts were filtered through paper filter and the residue again extracted with 100 mL of the respective solvents for 1 h under agitation. Both fractions were then blended and evaporated to dryness at 38°C under vacuum. Soxhlet method was carried out in a soxhlet apparatus with 15 g for 5 h extraction with the solvents A, B and C, resulting in the extracts 3A, 3B and 3C. The extracts were dried to dryness at 38°C under vacuum. All extracts were stored in a domestic freezer at -20°C until analysis.

Antimicrobial activity

Minimal inhibitory concentration (MIC) tests were carried out according to the NCCLS (National Committee of Laboratory Standards, 2003) (31) using a tissue culture test plate (96 wells). *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (isolated from swine), *Salmonella typhimurium* (ATCC 14028),

Pseudomonas aeruginosa (ATCC 13388) and *Clostridium perfringens* (ATCC 1324) were used in the tests. The microorganisms were grown overnight at 36°C in nutrient agar plates under aerobiosis, except for *Clostridium perfringens*, cultivated in thioglycollate broth under anaerobiosis. The inoculum for the assays was prepared by diluting scraped cell mass in 0.85% NaCl solution, adjusted to the McFarland scale 0.5. Cell suspensions were finally diluted to 10⁴ CFU mL⁻¹ for the assays. The extracts and the antibiotics were diluted in DMSO and stored at -20°C until used. The final concentrations of the extracts were 1.8, 1.5, 1.2, 1.0, 0.8, 0.6, 0.4 and 0.2 mg mL⁻¹, whereas serial dilutions of the antibiotics were prepared between 0.25 and 0.00195 mg mL⁻¹. The inoculum was added to all wells and the plates were incubated at 36°C for 24 h. To verify the sterility of extracts and culture medium (Mueller-Hinton 2.1%), as well as the viability of microorganisms, a control test was done with all test plates. Antimicrobial activity was detected by adding 0.05 mL of an aqueous 0.5% triphenyl tetrazolium chloride (TTC) solution. MIC was defined as the lowest oil or extract concentration that visually inhibited growth, indicated by TTC staining (dead cells are not stained by TTC).

CAT analysis

Adult and cotyledonary-stage dried leaves, seeds and burrs were extracted with 50% methanol (100 mg to 5 mL) for 20 minutes in ultrasound and filtered in 0.5 mm membrane (FHLP13, Millipore). Plant extract samples were analyzed through direct

infusion ESI-MS using a syringe pump (Harvard Apparatus) at a flow rate of 10 µL min⁻¹. ESI-MS fingerprints and ESI-MS/MS in the negative ion mode were obtained using a hybrid high-resolution and high-accuracy (5 ppm) Micromass Q-TOF mass spectrometer. Capillary and cone voltages were set to -3000 V and -50 V, respectively, at a desolvation temperature of 100°C. One milliliter of each sample was added to 10 mL of a solution containing 70% (v/v) chromatographic grade methanol and 30% (v/v) deionized water and 5 µL ammonium hydroxide per milliliter. Fingerprint mass spectra were obtained in the *m/z* range between 650 and 920.

RESULT AND DISCUSSION

Table 1 shows the antimicrobial activity of *X. strumarium* extracts and antibiotics. There were no differences between *X. strumarium* extracts, except for *S. aureus* and *C. perfringens*, which were affected by the used solvent but not by the extraction method.

The extracting solvents ethyl acetate (C) and dichloromethane/chloroform (1:1) (D) were more effective than 80% ethanol (A) and 80% methanol (B). Since *S. aureus* and *C. perfringens* are gram-positive bacteria, some cell wall active compounds have probably concentrated in the non-polar fraction; however, further studies are needed to prove this statement. *S. typhimurium* and *C. perfringens* were more resistant to *X. strumarium* methanolic and ethanolic extracts than *E. coli* and *P. aeruginosa*.

TABLE 1. Minimal inhibitory concentration (MIC, mg mL⁻¹) of *Xanthium strumarium* extracts and antibiotics.

Extract/antibiotic	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. typhimurium</i>	<i>C. perfringens</i>
1A	0.400-0.600	0.400-0.600	0.400-0.600	0.600-0.800	0.600-0.800
1B	0.400-0.600	0.400-0.600	0.400-0.600	0.600-0.800	0.600-0.800
1C	0.200-0.400	0.400-0.600	0.400-0.600	0.600-0.800	0.400-0.600
1D	0.200-0.400	0.400-0.600	0.400-0.600	0.600-0.800	0.400-0.600
2A	0.400-0.600	0.400-0.600	0.400-0.600	0.600-0.800	0.600-0.800
2B	0.400-0.600	0.400-0.600	0.400-0.600	0.600-0.800	0.600-0.800
2C	0.200-0.400	0.400-0.600	0.400-0.600	0.600-0.800	0.400-0.600
2D	0.200-0.400	0.400-0.600	0.400-0.600	0.600-0.800	0.400-0.600
3A	0.400-0.600	0.400-0.600	0.400-0.600	0.600-0.800	0.600-0.800
3B	0.400-0.600	0.400-0.600	0.400-0.600	0.600-0.800	0.600-0.800
3C	0.200-0.400	0.400-0.600	0.400-0.600	0.600-0.800	0.400-0.600
Penicilin G	*	0.008-0.015	0.062-0.125	0.062-0.125	0.062-0.125
Enrofloxacin	*	*	*	0.004-0.008	*
Erythromycin	*	*	0.031-0.062	0.031-0.062	0.031-0.062
Sulfadiazine	0.062-0.125	0.004-0.008	0.062-0.125	0.062-0.125	0.062-0.125
Oxytetracycline	*	0.008-0.015	0.062-0.125	0.062-0.125	0.062-0.125
Lincomycin	*	0.031-0.062	0.031-0.062	0.031-0.062	0.031-0.062
Spiramycin	0.002-0.004	0.004-0.008	0.062-0.125	0.062-0.125	0.062-0.125
Amoxicillin	*	0.031-0.062	0.062-0.125	0.062-0.125	0.031-0.062
Tetracycline	*	*	0.031-0.062	0.031-0.062	0.008-0.015
Sulfanilamide	0.062-0.125	0.004-0.008	0.062-0.125	0.062-0.125	0.062-0.125

1: maceration; 2: dynamic maceration; 3: soxhlet; A: 80% ethanol; B: 80% methanol; C: ethyl acetate; D: chloroform/dichloromethane (1:1). * < 0.0019 mg mL⁻¹.

Xanthatin was found at small quantities in *X. strumarium* essential oil. This compound was isolated from *X. spinosum* L. extracts and was active against *Colletotrichum gloeosporioides*, *Trichothecium roseum*, *Bacillus cereus* and *S. aureus* (Ginesta-Peris et al., 1994). A previous study reported that cinnamic acid was effective against *E. coli*, *P. aeruginosa*, *S. aureus* and *Salmonella* sp. and MIC values were 1.0 mg mL⁻¹ for all strains (Chang et al., 2001). Cinnamic acid was found in *X. strumarium* extracts between 22 and 80 mg g⁻¹ dry extract (Scherer et al., 2008). Thus, the compounds xanthatin and cinnamic acid could have contributed to the antimicrobial property of *X. strumarium*.

Staphylococcus aureus was the most susceptible to the tested antibiotics, followed by *E. coli*. MIC values for *S. aureus* were below 0.0019 mg mL⁻¹ for penicillin G, enrofloxacin, erythromycin,

oxytetracycline, lincomycin, amoxicillin and tetracycline. Enrofloxacin was the most effective antibiotic against all tested strains, followed by tetracycline and erythromycin, respectively (Table 1). On the other hand, sulfanilamide and sulfadiazine showed the highest MIC values for *S. aureus*.

Duarte et al. (2005) proposed a classification for plant material based on MIC results: strong inhibitors when MIC is below 0.5 mg mL⁻¹; moderate inhibitors when MIC is between 0.6 and 1.5 mg mL⁻¹; and weak inhibitors when MIC is above 1.6 mg mL⁻¹. Thus, considering this classification, all *X. strumarium* extracts showed potential antimicrobial activity against the evaluated microorganisms (Table 1).

Figure 1A shows the ESI-MS fingerprint of *X. strumarium* seed. Note the ion of *m/z* 769 which corresponds to the deprotonated molecule of carboxyatractyloside. Its ESI-MS/MS (Figure 1B)

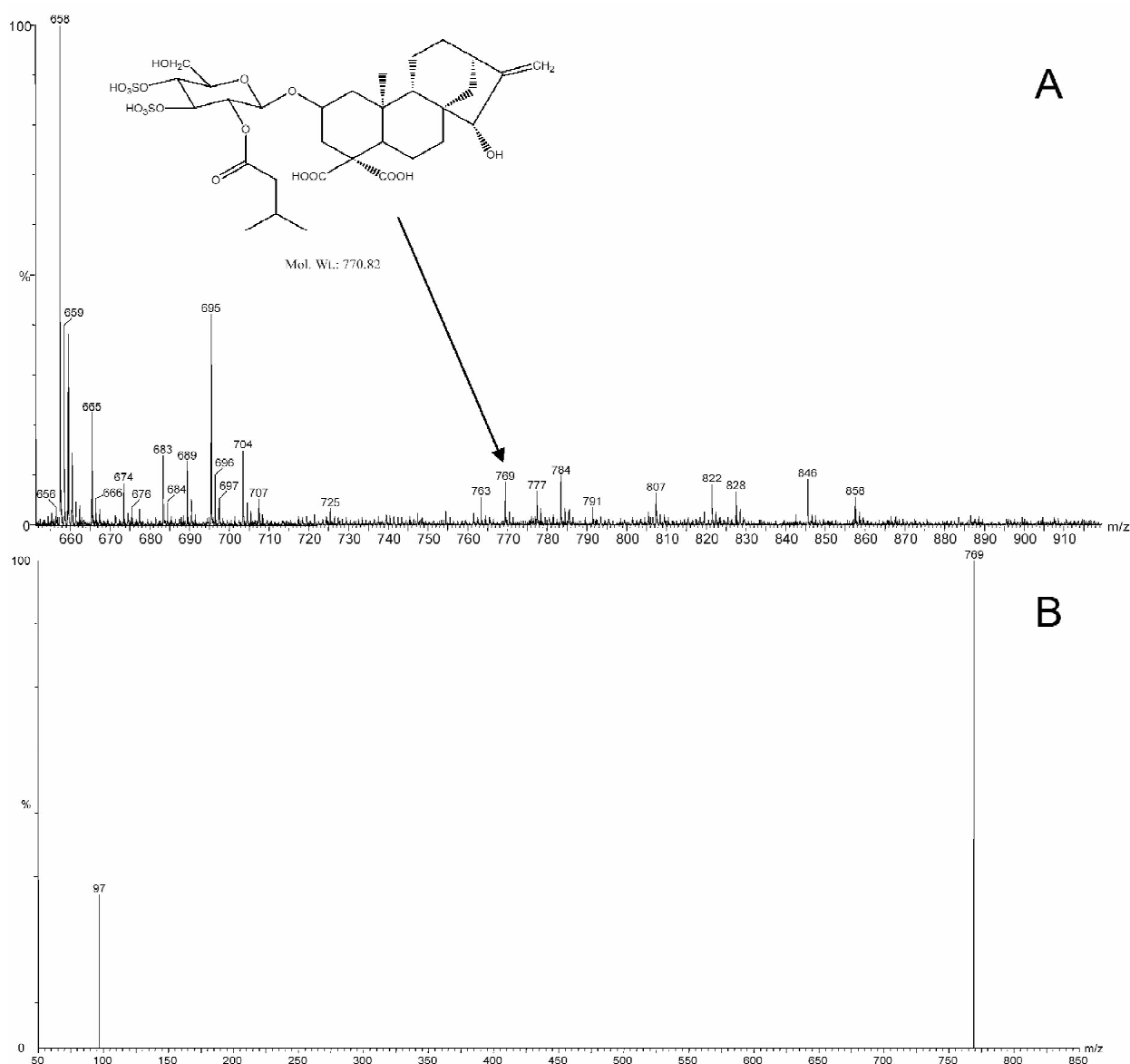


FIGURE 1. (A) ESI-MS of *Xanthium strumarium* seed extract and (B) ESI-MS/MS of deprotonated carboxyatractyloside.

confirms its structure since the ion is found to dissociate nearly exclusively into HSO_4^- of m/z 97. ESI-MS/MS monitoring indicated carboxyatractyloside was present in extracts from seeds and cotyledonary-stage leaves but not in those from adult leaves and burrs.

Carboxyatractyloside is toxic and was originally isolated from the Mediterranean thistle *Atractylis gummifera*, a highly selective inhibitor of the cytosolic side-specific mitochondrial ADP/ATP carrier (Huber et al., 1999). As already mentioned, previous studies have reported that *X. strumarium* induces liver damage and can be deathful; thus, care should be taken concerning the medicinal use of *X. strumarium* and only its leaves in adult stage are recommended, since the latter had strong antimicrobial activity and did not present the toxic compound.

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