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Journal of Medicinal Plants Research

ARTICLE

Full Length Research Paper

Antifungal activity and synergistic effect of acetophenones isolated from species *Croton* against dermatophytes and yeasts

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The aim of this study was to evaluate acetophenones isolated from *Croton* spp. for antifungal activity *in vitro* against *Candida albicans*, *Microsporum canis* and *Trichophyton rubrum*, and to evaluate the toxicity of these substances *in vivo*. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined using the broth microdilution method. The combined effect of the 2-hydroxy-3,4,6-trimethoxyacetophenone with ketoconazole was evaluated using the checkerboard technique. The toxicity was determined using *Artemia salina* L. The compounds were active against *T. rubrum*, with MICs ranging from 1.25 to 2.5 mg/mL. Similar results were obtained for *M. canis*, with MICs ranging from 5.0 to 10.0 mg/mL. In the toxicity tests, 2-hydroxy-4,6-dimethoxyacetophenone and 2-hydroxy-3,4,6-trimethoxyacetophenone presented LC_{50} of 46.22 and 75.55 µg/mL, respectively. 2-hydroxy-3,4,6-trimethoxyacetophenone exhibited a synergistic effect with ketoconazole.

Key words: Checkerboard, *Croton* spp., fungal infections, *Microsporum canis*, *Trichophyton rubrum*.

INTRODUCTION

Plants have been in use for therapeutic purposes in many nations and are still a widely acceptable alternative treatment method not only in urban centers, but also in small rural communities. Among plant families

comprising the world's flora, Euphorbiaceae is one of the largest groups of dicotyledons, containing about 300 genera and 5,000 species. In Brazil, there are 72 genera and around 1,300 species, with *Croton* being the second largest genus, comprising approximately 700 species (Salatino et al., 2007).

Phytochemical studies of *Croton* species have identified the presence of terpenoids (Barreto et al., 2013; Sousa et al., 2015), alkaloids (Risco et al., 2003; Araújo-Junior et al., 2004), flavonoids (Zou et al., 2010; Barreto et al., 2013), triterpenes and steroids (Catalán et al., 2003; Maciel et al., 2006). These secondary metabolites are responsible for a wide variety of pharmacological activities against fungi and bacteria (Salatino et al., 2007; Carneiro et al., 2011). These pharmacological activities are of great importance because the usual antimicrobial agents are becoming less effective, especially due to the fungi and bacterial resistance caused by the widespread use of these drugs (Sá et al., 2012). Research into new substances with antimicrobial activity can make a major contribution to human health worldwide, by finding a more efficient and less toxic antimicrobial substances in the fight against pathogenic microorganisms' resistance (Saúde-Guimarães et al., 2007).

Cutaneous mycoses are among the most common fungal infections in humans and are mostly caused by dermatophytes which are keratinophilic filamentous fungi belonging to the genera *Trichophyton*, *Microsporum* and *Epidermophyton* (Havlickova et al., 2008; Guo et al., 2012). These fungi are cosmopolitan and according to the World Health Organization (WHO), they affect 25% of the world population. Till date, about 30 species have been identified as pathogenic to humans. It is estimated that about 30 to 70% of adults are asymptomatic host of these pathogens and that the incidence of mycoses is influenced by the individual's age (Peres et al., 2008; White et al., 2008; Seebacher et al., 2010).

In recent years, the prevalence of infections caused by *Candida* species (candidiasis) has been on the increase. In fact, these species are now ranked as the fourth leading cause of infections. This increase in infections caused by *Candida* is generally explained by the growing use of medical devices such as implants, prostheses and catheters, excessive use of antimicrobial agents, general aging of the population and the rising number of immunocompromised patients (Negri et al., 2012).

There are many literature reports on the isolation of fungal strains that are resistant to the antifungal drugs available in the market (Posteraro et al., 2006; Brito et al., 2007; Yenisehirli et al., 2007; Eksi et al., 2013). This fact together with the high cost of most antifungals has prompted the search for effective alternative therapies to increase the number of treatment options and to find a more effective and less expensive compounds. In this context, there is the need to find natural products with antimicrobial properties and phytotherapeutic potential (Mehraboni et al., 2013).

The phytochemical study of *Croton anisodontus* and *Croton nepetaefolius* led to the isolation of two compounds; 2-hydroxy-3,4,6-trimethoxyacetophenone and 2-hydroxy-4,6-dimethoxyacetophenone, respectively (Figure 1). These acetophenones are phenolic compounds with biological effects such as anti-inflammatory (Sala et al., 2001; Malaviya et al., 2010), antispasmodic (Cechinel Filho et al., 1995; Niero et al., 1996), antiproliferative (Pisco et al., 2006) and antimicrobial (Bonifait et al., 2012).

The aim of this study was to assess the modulatory and antifungal activities of acetophenones isolated from *Croton spp.* against strains of dermatophytes and yeasts.

MATERIALS AND METHODS

Plant material

The plant material (stems) of *C. anisodontus* Müll.Arg. and *C. nepetaefolius* Baill. were collected in the city of Itapiuna, Ceará State, Brazil, in March 2011, at coordinates 04° 33' 50" S and 38° 55' 19" W, and in the city of Caucaia, Ceará, Brazil, respectively. The botanical identification was performed by Professor Edson Nunes of Ceará Federal University (UFC). Voucher specimens of the species were deposited in the Prisco Bezerra Herbarium of UFC under numbers 48.964 and 33.582, respectively.

Experimental

Adsorption column chromatography was carried out with silica gel 60 (63 to 200 μm, 70 to 230 mesh, Vetec). Column length and diameter were varied according to the sample amount. TLC used aluminum chromatoplates (20 x 20 cm) with silica gel 60 GF$_{254}$ (Merck). Substances on the plates were revealed by exposure to a vanillin solution followed by heating. Infrared spectra were obtained with a Perkin-Elmer 1000-FT spectrometer. Melting points were determined with a Mettler Toledo digital microdetermination apparatus. One and two dimensional nuclear magnetic resonance (NMR) spectra were obtained on a Bruker DRX-500 (^{1}H: 500 MHz; ^{13}C: 125 MHz, respectively) spectrometer, using CDCl$_3$ as solvent and TMS as the internal standard. Mass spectra (MS) were recorded with a Shimadzu QP5050A spectrometer, operating at 70 eV.

Extraction and isolation of acetophenones

C. nepetaefolius stems (5.0 kg) were powdered and subjected to extraction using ethanol (10 L×3, at room temperature). The solvent was removed under reduced pressure to give EtOH extract. The EtOH extract (58.2 g) was coarsely fractionated in a silica gel column by elution with *n*-hexane (F 1 to 15), *n*-hexane/EtOAc 1:1 (F 16 to 25), EtOAc (F 26 to 40), and EtOH (F 41 to 48), resulting to a total of 48 fractions of 100 mL each. The fractions of *n*-hexane/EtOAc 1:1 (F 16 to 25; 10.8 g) were pooled and fractionated in a silica gel column with *n*-hexane (F′1), *n*-hexane/EtOAc (9:1 F′2 to10; 7:3 F′11 to 13; 1:1 F′14 to 15) and EtOAc (F′16), producing 16 fractions of 100 mL each. The fractions F′ 10 to 13 and F′16 obtained using *n*-hexane/EtOAc (7:3) and EtOAc yielded white crystals [mp 77 to 78.5°C, MS (70 eV, in percent) m/z 196 [M]+, 181 (100), 166 (15), 151 (8), 138 (23), 95 (8)], whose ^{1}H and ^{13}C NMR spectra and other properties coincided with the published values for 2-hydroxy-4,6-dimethoxyacetophenone

(Santos et al., 2008).

C. anisodontus stems (1462.6 g) were dried at room temperature, followed by trituration and cold extraction with *n*-hexane for 3 days. The resulting solution was distilled under reduced pressure to give the EtOH extract (23.25 g), which was adsorbed onto silica gel and eluted through a chromatographic column, using the following eluents: *n*-hexane, chloroform, ethyl acetate and ethanol. The chloroform fraction (9.77 g) was reintroduced to the column and eluted with: *n*-hexane (F 1 to 6), *n*-hexane/ethyl acetate (8:2 F'7 to 26), *n*-hexane/ ethyl acetate (7:3 F 27 to 40), ethyl acetate (F 41 to 60) and methanol (F 61 to 66). Fractions 12 to 16 revealed a yellow crystalline solid; 2-hydroxy-3,4,6-trimethoxyacetophenone, which after recrystallization with *n*-hexane and analysis by thin layer chromatography was found to be pure (Oliveira et al., 2014).

Bioassay with *Artemia salina* L.

Toxicity tests were conducted according to the method proposed by McLaughlin (1991), where serial dilutions were carried out from 20 mg of the samples with chloroform ($CHCl_3$) to obtain a final concentration of 1,000, 100, 10 and 1 ppm. The negative control was 100 µl of 2% dimethyl sulfoxide (DMSO) and 4.9 mL of distilled water.

0.5 mL of each sample was added to all test tubes with 1.0 mL saline and 50 µl of DMSO. The tubes were placed in a sonicator for 10 min with the aid of a pipette. 48 h after hatching, 10 *Artemia salina* L. larvae were transferred to each test tube. The volume of the tubes was completed with saline to 5 mL. The assays were performed in triplicate.

The tubes were incubated for 24 h, after which the number of dead microcrustaceans was counted to compute the mortality percentage. The results were tabulated and submitted to probit analysis using SPSS 10.0 for Windows, to obtain the LC_{50} value with a confidence interval of 95%.

Fungal strains

The strains of *Trichophyton rubrum* and *Candida albicans* were obtained from the fungal collection of the Specialized Medical Mycology Center (CEMM, Federal University of Ceara, Brazil). The strains of *T. rubrum* were isolated from symptomatic patients and those of *C. albicans* were isolated from cats. *M. canis* strains were isolated from dogs. All the strains were stored in the microbiology laboratory (Vale do Acaraú State University, Brazil), where they were maintained in saline (0.9% NaCl) at 28°C. At the time of the analysis, an aliquot of each suspension was taken and inoculated into potato dextrose agar (Difco, Detroit, USA), and then incubated at 28°C for about 2 to 10 days respectively. The identification of dermatophytes was based on phenotypic features, such as a description of the macro and micromorphology. The pigment production and sporulation of dermatophytes were considered as well as the production of the enzyme urease to differentiate species of the genus *Trichophyton* (Vasconcellos et al., 2013; Mattei et al., 2014). The *C. albicans* strains were identified by automated identification (Vitek system platform) (Durham, USA).

Inoculum preparation for antifungal susceptibility tests

The inoculum was prepared from strains grown on Sabouraud dermatophytic agar for 5 days at 35°C and yeast strains for 24 h at 35°C. Fragments of *M. canis*, *T. rubrum* and *C. albicans* were transferred to tubes containing sterile saline solution (0.85%) to obtain a turbidity equivalent to standard (5x106 CFU/mL or 0.5) on the McFarland scale.

The suspensions were diluted to 1:2000 for *C. albicans* and 1:500 for *M. canis* and *T. rubrum,* both with RPMI 1640 medium with L-glutamine, without sodium bicarbonate (Sigma Chemical Co., St. Louis, Mo.), and buffered to pH 7.0 with 0.165 M morpholine-propanesulfonic acid (MOPS) (Sigma Chemical Co., St. Louis, Mo.), to obtain inoculum concentrations of approximately 2.5 to 5 x 10^3 CFU/mL and 5 x 10^4 CFU/mL respectively.

Broth microdilution method

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of acetophenones against the dermatophyte and yeast strains were determined by the broth microdilution method using 96-well plates. The MIC for *C. albicans* was determined in accordance with the Clinical and Laboratory Standards Institute-CLSI (formerly NCCLS; M27-A2) (CLSI, 2008). The broth microdilution assay for *M. canis* and *T. rubrum* was performed based on the document M38-A (CLSI, 2002).

The compounds were prepared in DMSO at a concentration of 10 mg/mL diluted in 100 µL of RPMI 1640 medium (Sigma) and tested in a concentration range of 0.009 to 10.0 mg/mL. Thereafter, a 100 µL inoculum was added to the plates to produce a final volume of 200 µL per well. The antifungal ketoconazole for dermatophytic strains and amphotericin B for yeast strains, with a range from 0.07 to 16 µg/mL, were used as controls. The microplates were incubated at 37°C and read visually after 5 days. *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6528) were included as quality controls. The MIC was defined as 100% inhibition of visible fungal growth. Consecutively, the minimum fungicidal concentration was determined after the transfer of 100 µL of the contents of the well without turbidity into tubes containing potato dextrose agar at 28°C. The MFC against dermatophytes was calculated in accordance with fungal growth in the culture medium after 5 days, whereas for the yeasts it was determined after 24 h. Each experiment was run in duplicate.

Microdilution checkerboard assay

The effect of the compounds combined with commercial antifungal was determined by the checkerboard technique, a method used to determine the interaction of the drug by calculating the fractional inhibitory concentration index (FICI). The FICI is calculated by summing the fractional inhibitory concentration (FIC) for each of the tested compounds, which in turn is determined by summing the MIC of each drug in combination divided by the MIC of the same drug alone.

The turbidity of the fungal suspensions was adjusted to 0.5 on the McFarland scale (10^5 CFU/mL). Solutions of the products tested in certain concentrations from their respective MIC values were used. Initially, 50 µL of RPMI medium was added to all 96 wells of microdilution plates. Then 50 µL of the compound was added in the first column, in which serial dilutions were made in the plate until the 8th column, with the compound concentrations ranging from 10.0 to 0.07 µg/mL. In the vertical lines, 50 µL of standard antifungal ketoconazole was placed in concentrations ranging from 16 to 0.125 µg/mL. Finally, 100 µL of inoculum was added to all wells. RPMI 1640 medium with inoculum was used as a negative control, while the ketoconazole and acetophenone were separately used as positive controls at the respective MIC values. The dermatophyte plates were incubated at 36°C for 5 days. Assays were performed in triplicate.

The FICI was calculated according to the equation below, where A represents acetophenone and B the antifungal ketoconazole:

FIC_A = MIC of agent A in combination / MIC of agent A alone

FIC_B = MIC of agent B in combination / MIC of agent B alone

Table 1. Minimum inhibitory concentration (MIC) of the acetophenones against the dermatophytes and yeasts.

Strains	1		2		Amphotericin B		Ketoconazole	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Candida albicans								
CEMM 01-3-068	NI	NI	NI	NI	0.001	0.002	-	-
CEMM 01-3-069	NI	NI	NI	NI	0.002	0.004	-	-
Trichophyton rubrum								
CEMM 5-1-34	2.5	5.0	2.5	5.0	-	-	0.004	0.008
CEMM 5-1-08	1.25	2.5	2.5	5.0	-	-	0.002	0.004
Microsporum canis								
LABMIC 1-1-01	10.0	-	5.0	10.0	-	-	0.004	0.008
LABMIC 1-1-02	10.0	-	5.0	10.0	-	-	0.002	0.004

(1) 2-hydroxy-4,6-dimethoxyacetophenone; (2) 2-hydroxy-3,4,6-trimethoxyacetophenone; MIC: Minimum Inhibitory Concentration, expressed in mg/mL; MFC: Minimum Fungicidal Concentration, expressed in mg/ml; NI: no inhibition. LABMIC: Microbiology Laboratory; CEMM: Specialized Centre of Medical Mycology.

Table 2. Synergism results of 2-hydroxy-3,4,6-trimethoxyacetophenone and ketoconazole against *Trichophyton rubrum* and *Microsporum canis* dermatophytes

Combinations	*Trichophyton rubrum*		*Microsporum canis*	
	FIC (µg/mL)	FICI (µg/mL)	FIC (µg/mL)	FICI (µg/mL)
2-hydroxy-3,4,6-trimethoxyacetophenone	0.03	0.06	0.06	0.31
Ketoconazole	0.03		0.25	

FIC- Fractional Inhibitory Concentration; FICI- Fractional Inhibitory Concentration Index; Interpretation of values- synergism (FICI ≤ 0.5); indifferent (FICI> 0.5 and ≤ 4.0); antagonism (FICI> 4.0).

$$FICI = FIC_A + FIC_B$$

The FICI indicated a synergistic effect at values ≤ 0.5, an indifferent effect at values > 0.5 or ≤ 4.0, and an antagonistic effect at values > 4.0 (Odds, 2003; Johnson et al., 2004).

RESULTS

Bioassay with *Artemia salina* L.

In the toxicity tests, 2-hydroxy-4,6-dimethoxy-acetophenone and 2-hydroxy-3,4,6-trimethoxy-acetophenone presented LC_{50} of 46.22 µg/mL with an interval of 29.22 to 73.37 and LC_{50} of 75.55 µg/mL with an interval of 46.55 to 125.47, respectively. These results indicated that both compounds are bioactive, with the former being more toxic than the latter, because the former compound required a lower quantity to cause 50% mortality of the population.

MIC and MFC determination

The data presented in Table 1 shows that the acetophenones tested have similar antifungal activity against the dermatophytes. 2-hydroxy-4,6-dimethoxy-acetophenone was active against *T. rubrum*, with MICs ranging from 1.25 to 2.5 mg/mL and MFCs of 2.5 to 5.0 mg/mL for the two strains tested, while 2-hydroxy-3,4,6-trimethoxyacetophenone presented MIC of 2.5 mg/m and MFC of 5.0 mg/mL against the same strains.

For the strains of *M. canis*, 2-hydroxy-4,6-dimethoxy-acetophenone presented MIC of 10.0 mg/mL at the highest concentration tested, but did not present MFC. The yeast strains were not sensitive to either of the two compounds.

Synergistic effect of ketoconazole

To assess the possibility of a more powerful antifungal effect of acetophenone, pooled samples were tested in combination with the commercial antifungal agent. Table 2 demonstrates a synergistic effect of ketoconazole in combination with acetophenone, with MIC values just below four-fold of the individual compounds.

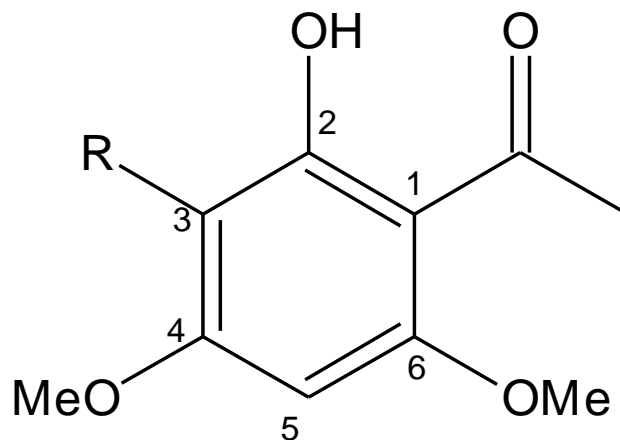The startling combination effects of ketoconazole and

Figure 1. Structural formula of acetophenones isolated from *Croton* species. **[1] R = H [2] R = OMe.**

the compound were confirmed in this test, with FICI of 0.06 µg/mL against T. rubrum and FICI of 0.31 µg/mL against M. canis, resulting in synergic effects.

DISCUSSION

Meyer et al. (1982) established a relation between the degree of toxicity and the mean lethal dose (LC_{50}) of plant substances against larvae of *A. salina* L., and since then the benchmark has been that LC_{50} values <1000 µg/mL indicate that a substance is bioactive. This result corroborates the antifungal properties of the compounds.

As a result of the resistance developed by micro-organisms to drugs currently used to treat fungal infections, there is the need to find new compounds with biological activity, particularly those of plant origin. The two compounds tested here; 2-hydroxy-4,6-dimethoxy-acetophenone and 2-hydroxy-3,4,6-trimethoxy-acetophenone, presented antifungal activity against the dermatophyte strains investigated.

Despite the large number of articles reporting experiments to find compounds with antifungal activity, only a few have reported the action mechanism of the compounds tested (Zacchino, 2001). Therefore, the broth microdilution method was used in this study, but it was not possible to determine the action mechanism of the acetophenones against the fungal strains studied. However, it is believed in this study that it could be related to the synthesis of ergosterol, since there are reports in the literature of acetophenones that have hypocholesterolemic activity associated with synthesis of this sterol (Piyachaturawat et al, 2002).

Despite having activity against strains of dermatophytes, the compounds were not active against strains of *C. albicans*. Resistance of *Candida* strains is related to

several virulence factors, which comprises adhesion and biofilm formation, dimorphism, cell wall composition and secretion of hydrolytic enzymes such as proteases and phospholipases (Giolo and Svidzinski, 2010; Sardi et al., 2013).

Studies of antifungal activities of the acetophenones from plants are still scarce in the literature. Gul et al. (2002) reported good antifungal activity of acetophenone-derived bis Mannich bases against the dermatophyte species of *T. rubrum* and *M. canis*, with potential for developing novel antifungal agents.

In a study of the antifungal activity of acetophenone derivatives, 2,3,4 trihydroxyacetophenone (Gala) was the compound that best inhibited the filamentous fungi, with MIC of 31.2 µg/mL, indicating greater activity than fluconazole against *M. canis*. On the other hand, 2,4,6-trihydroxyacetophenone (THA) was the least active compound against the yeast *C. albicans*, presenting MIC of 125µg/mL (Rebelo, 2005). The results of this study demonstrated the oxidative potential of THC besides the antioxidant activity *in vitro* of the two acetophenones tested.

The oxidative potential of THA was also demonstrated by Ferreira (2005) in an investigation of the oxidative activity of synthetic analogs of acetophenone, where THA and Gala also showed important oxidative potential, *in vitro* and *in vivo*. THA also presented strong hypertriglyceridemic activity, acting as an excellent inhibitor of absorption of triglycerides in animals treated with olive oil.

The activity *in vitro* of compounds with little or no antifungal activity has been tested in combination with antifungal drugs (Khan and Ahmad, 2012). Therefore, the interaction between the compounds and antifungal drugs may prove to be an alternative for the treatment of fungal infections and antifungal resistance (Vandeputte et al.,

2012). The compound 2-hydroxy-3,4,6-trimethoxy-acetophenone in combination with the antifungal ketoconazole *in vitro* had a synergistic effect, thus enhancing the antifungal activity of the compound, suggesting future pharmacological use as an adjuvant for these drugs (Guerra et al., 2015).

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Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Leaves of *Schinus polygamous* (Cav.) Cabrera (Anacardiaceae) are a potential source of hepatoprotective and antioxidant phytochemicals

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Phytochemical investigation of the crude ethanol extract of the leaves of *Schinus polygamus* (Cav.) Cabrera (Anacardiaceae) results in isolation of eight compounds identified as triterpenoids, 3-O-acetyllupeol (I), *βeta*-sitosterol (II), lupeol (III). In addition to the polyphenols, gallic acid (IV), methyl gallate (V), quercetin-3-*α-O*-rhamnoside (VI), kaempferol (VII) and quercetin (VIII). In Egypt, degenerative diseases in general and toxic hepatitis in particular remain a serious public health problem. The hepatoprotective, curative and anti-oxidant properties of the major phytochemicals, compounds III and IV isolated from leaves of *S. polygamus* were investigated. Liver damage was induced by CCl_4 (1 mlkg^{-1}); a well-known toxicant and exposure to this chemical is known to induce oxidative stress and causes tissue damage by the formation of free radicals. Silymarin (25 mgkg^{-1}) and vitamin E (7.5 mgkg^{-1}) were used as standard drugs to compare hepatoprotective and antioxidant effects of the phytochemicals, respectively. Oral administration of 50 to 100 mgkg^{-1} body weight of compounds III and IV were significantly protected from CCl_4 induced elevation in aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in adult male albino rats. The antioxidant effect in the liver was estimated by measuring the activity of antioxidant enzyme reduced glutathione. Detection of gallic acid and lupeol in *S. polygamus* as a member of family Anacardiaceae support the claim that both compounds could be considered as chemotaxonomic markers for plants belong to family Anacardiaceae. Results of the present study strongly reveal that both compounds III and IV have potent antioxidant and hepatoprotective effects against CCl_4-induced hepatic intoxication.

Key words: *Schinus polygamus* (Cav.) Cabrera, Anacardiaceae, triterpenes, phenolics, hepatoprotective, antioxidant effect.

INTRODUCTION

Metabolic processes of the body produce significant amounts of reactive oxygen species (ROS). Liver is a major organ attacked by ROS. Damaging effects brought by these ROS are being counteracted by the cellular

antioxidant defense system of the body (Hopps et al., 2010; Santiago and Mayor, 2014). However, at certain conditions, oxidative stress is triggered due to the imbalance between the production of ROS and the antioxidant systems of the body (Pepe et al., 2009). High risk health conditions such as neurodegenerative diseases, hepatitis, diabetes, cancer and inflammation are usually related to oxidative stress (Fearon and Faux, 2009; Fang et al., 2002). Based on growing interest in free radical biology and lack of effective therapies for most chronic diseases, the usefulness of antioxidants in protection against these diseases is warranted (Huang et al., 2010). In this regard, phytochemicals from various natural products had become the subject of most drug development researches (Santiago and Mayor, 2014). Plants are the most commonly known sources of natural antioxidants which includes polyphenolic and terpenoids compounds (Grassmann, 2005; Dimitrios, 2006).

Family Anacardiaceae (Bailey, 1953) has long reputation in folk medicine for its nutritional value of edible fruits and seeds, and for variable ailments such as treatment of bowel complain, chronic wounds, jaundice, hepatitis and relieving inflammatory conditions (Abbasia et al., 2010). There is an extensive body of literature addressing the escalated distribution of hepatic diseases among the people in Egypt (Shabana et al., 2011; Lehman, 2008; Strickland et al., 2002). Hepatoprotective herbal drugs can offer help by blocking absorption of toxins into liver cells and the formation of inflammatory substances that contribute to liver degeneration (Shabana etal., 2011).

As part of on-going study of medicinal plants belongs to family Anacardiaceae, the closely related genera, *Harpephyllum caffrum* Bernh, *Rhus coriaria* L and *Schinus polygamus* Cav. were subjected to biological testing in order to confirm the claimed herbal benefits of these drugs by different communities (Van Wyk et al., 2009; Rayne and Mazza, 2007; Shabana et al., 2008, 2011, 2013).

Schinus polygamus (Cav.) Cabrera, family Anacardiaceae is a tree cultivated for ornamental purpose. Traditionally the leaves have been used for wounds cleansing, while the bark produces a balsamic resin, is used to treat arthritic and chronic bronchitis (Munoz et al., 1981). The presence of well-known flavonoids was reported in *S. polygamus*, namely, kaempferol, quercetin and quercetin-3-*O*-galactoside (Mandich et al., 1984). The *n*-hexane extract of *S. polygamus* were reported to contain *β*-sitosterol to which analgesic, anti-inflammatory and antipyretic activities of the Argentine and the Chilean species were attributed (Erazo et al., 2006; Gonzalez et al., 2004). The essential oil obtained from *S. polygamus* leaves exhibited antimicrobial activities (Gonzalez et al., 2004).

Nowadays, the current research on plant based medications focus on isolation of biologically active substances from potent plants (Kingston, 2011).

However, nothing could be traced on phytochemicals of Egyptian *S. polygamus* and its biological potentiality. Considering the health benefits one can derive from *S. polygamus* (Mandich et al., 1984; Munoz et al., 1981), its phytochemical, biochemical and pharmacological studies were scarce. Moreover, chemical profiling of the antioxidative components present in the plant is not yet been fully elucidated. Therefore this study was undertaken to back up its ethnobotanical uses while intensively evaluating the antioxidative components of *S. polygamus* as well as assessing its potential role as a powerful antioxidant and hepatoprotective

MATERIALS AND METHODS

Plant

The leaves of *S. polygamus* (Cav.) Cabrera were obtained from trees growing in El Orman botanical garden, Giza in April 2013. The plant was kindly authenticated by Dr. Mohamed El-Gebaly, Botany Specialist. Voucher specimen 1842013 is kept in the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

Preparation of the extracts

Two kilograms of air-dried powdered leaves of *S. polygamus* were extracted by cold percolation with 90% ethanol (5 × 1 L). The ethanol extract was evaporated under reduced pressure to give 350 g green semi-solid residue. 250 g of the crude extract were successively partitioned between *n*-hexane, chloroform, ethyl acetate and *n*-butanol saturated with water. The solvent in each case was completely evaporated under reduced pressure to yield 30, 10, 150 and 3 g, respectively.

Material for phytochemical study

For chromatographic studies pre-coated silica plates 60 GF $_{254}$, cellulose plates (20×20 cm) from Fluka (Sigma-Aldrich chemicals-Germany) for thin layer chromatography (TLC), silica gel 60 for normal phase column chromatography (CC), silica gel H for vacuum liquid chromatography (VLC) (E-Merck Darmstadt, Germany) polyamide (E-Merck Darmstadt, Germany), sephadex LH 20 and silica gel RP-18 (70 to 230 mesh) for reversed phase column chromatography were obtained from Fluka (Sigma-Aldrich chemicals-Germany). For developing the chromatograms, the following solvent systems were used: S1=*n*-hexane: chloroform (9:1 v/v), S2=*n*-hexane:chloroform (7:3 v/v), S3=*n*-hexane:ethylacetate (6:4 v/v), S4=*n*-butanol:acetic acid: water (4:1:5 v/v/v), S5=benzene: ethyl acetate: formic acid (5.5:4.5:1 v/v/v) and S6=ethyl acetate: methanol: formic acid: water (8:2:0.5:1 v/v/v/v). Spots were visualized by spraying with the following spray reagents: (I) *p*-anisaldehyde-sulphuric acid for triterpenoids, (II) 1% aluminium chloride spray reagent for flavonoids, (III) ferric chloride spray reagent for phenolic compounds (Mabry et al., 1996).

Apparatus for phytochemical study

Ultraviolet lamp (λ$_{max}$=254 and 330 nm), Shimadzu, a product of Hanovia lamps for localization of spots on chromatograms. EI-MS

were recorded with a Varian Mat 711, Finnigan mass SSQ 7000 Mass spectrometer, 70 eV. IR spectra were observed as KBr discs on Shimadzu IR-435, PU-9712 infrared spectrophotometer. ^{1}HNMR (300 MHz) and ^{13}CNMR (75 MHz) spectra were recorded on Jeol EX-300MHz and Bruker AC-300 spectrometer operating at 300 (^{1}H) and 75 (^{13}C) MHz in $CDOD_3$ and $CDCl_3$ as a solvent and chemical shifts were given in δ (ppm).

Phytochemical investigation of the n-hexane soluble fraction

Thirty grams of the *n*-hexane soluble fraction were chromatographed on a VLC column, 210 g silica gel (12.5 × 7 cm) using *n*-hexane, *n*-hexane-chloroform and chloroform-ethyl acetate mixtures as elutes. Fractions, 200 ml each, were collected and monitored by TLC. Similar fractions were pooled together to obtain three major fractions (A-C).

Fraction A (4.0 g), eluted with *n*-hexane, was rechromatographed on silica gel sub-column using *n*-hexane and1% chloroform in *n*-hexane as solvent systems to obtain compound I (0.02 g).

Fraction B (2.5 g), eluted with 15% chloroform in *n*-hexane, was purified on silica gel column using *n*-hexane-chloroform mixtures (10% chloroform in *n*-hexane) as an eluent to obtain compound II (0.03 g).

Fraction C (2.0 g), eluted with 35% chloroform in *n*-hexane, was purified on silica gel column using *n*-hexane-chloroform mixtures (80:20 and 75:25), respectively as eluents revealed two spots. Further rechromatography on successive silica gel columns using *n*-hexane-chloroform mixtures yielded compounds III (0.25 g).

Phytochemical investigation of the ethyl acetate soluble fraction

Ethyl acetate soluble fraction (150 g) was purified on column chromatography (300 g polyamide, 50 × 3 cm) using 100% water and water-methanol mixtures in order of decreasing polarity. Fractions, 100 ml each, were collected and monitored by TLC. Similar fractions were pooled together to obtain two major fractions (a,b).

Fraction a (2 g), eluted with 15 and 25% methanol in water revealed the presence of three major spots. Further re-chromatography using sephadex LH 20 and water-methanol in decreasing polarity as eluent led to the isolation of two phenolic compounds IV and V (0.5 and 0.08 g), respectively.

Fraction b (5.37 g), eluted with 30 to 50% methanol in water, was further subjected to rechromatography on RP silica column using water- methanol as elute and resulted in separation of three compounds VI (0.03 g), VII (0.05 g) and VIII (0.03 g).

Material for biological study

The tested solutions were prepared by dissolving them in distilled water containing few drops of Tween 80 to yield a concentration of 5% w/v.

Animals

Adult Sprague-Dawely rats weighing 160 ± 10 g were provided from the animal-breeding unit of National Research Center, El-Dokki, Giza, Egypt. They were kept under standard conditions with temperature at 23 ± 2°C and a 12/12 h light/dark cycle and allowed free access to food and water throughout the experiment. This study was conducted in accordance with the standard guidelines used in handling of the experimental animals and approved by the Institutional Animal Care and Use Committee (IACUC) (No. 9-031), College of Pharmacy, Cairo University.

Drugs and chemicals

Carbon tetrachloride (Analar, El-Gomhoreya Co., Cairo, Egypt, for induction of liver damage; 5 ml kg^{-1} of 25% carbon tetrachloride in liquid paraffin, IP) (Paget and Barnes, 1964) (Silymarin (Sedico Pharmaceutical Co., 6 October City, Egypt, standard hepato-protective drug; 25 mgkg[-1] b.wt.), Biodiagnostic kits for assessment of serum alanine aminotransferase (ALT), aspartate amino-transferase (AST) and alkaline phosphatase enzymes (ALP) estimated kits were purchased from Bio-Merieux Co. (France). Vitamin E (7.5 mgkg[-1] b.wt.) (dlα-tocopheryl acetate): Pharco Pharmaceutical Co. Biochemical kits: Biodiagnostic glutathione kit.

Experimental design

For testing the hepatoprotectiveeffect on the liver, 36 adult male albino rats were used in the experiment and divided into six groups (each of 6). Group served as control, received saline (1 ml kg^{-1}/day) and received CCl_4 1 mlkg[-1](1:1 CCl_4 in liquid paraffin orally) once daily for 7 days. Group received CCl_4 1 mlkg[-1] and silymarin 25 mgkg[-1] orally for 7 days as a reference drug. Four groups were administered the tested compounds at a daily dose of 50 and 100 mgkg-[1] b.wt. (Paget and Barnes, 1964). The compounds, as well as, the reference drug, were administered to the rats for another seven days after liver damage.

For testing the antioxidant effect on liver, five groups of animals (6 animals each) were divided. One group was kept as a control, while for the other groups, group with liver injury was kept non-treated, another group received daily the reference drug (Vitamin E) and the other groups received the tested isolates of *S. polygamus* daily in the given doses. Blood samples were taken after 7 days.

Estimation of hepatoprotective activity

Measurement of AST, ALT and ALP serum levels

The animals were scarified and blood sample was collected from the retro-orbital sinus plexus under mild ether anesthesia. Collected blood was allowed to clot and serum was separated at 3500 rpm for 15 min for carrying out biochemical investigation serum levels of aspartate aminotransferase (AST) (Thewfeld, 1974), alanine aminotransferase (ALT) (Thewfeld, 1974), and alkaline phosphatase (ALP) (Kind and King, 1954; Klassan and Plaa, 1969) enzymes were measured in each group at zero time, after 7 days of receiving the tested drug, 72 h after induction of liver damage and after 7 days of treatment with the tested samples.

Estimation of antioxidant activity

The antioxidant activity was calculated by the determination of glutathione in blood of CCl_4-induced liver injury rats adopting the methods of (Beutler, 1975; Beutler et al., 1963) using vitamin E as a reference drug. Blood samples were taken after 7 days for the determination of glutathione.

Statistical analysis

All data were expressed as mean ± standard error (SE). The statistical comparison of difference between the control group and the treated groups were carried out using two-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

RESULTS

Phytochemical investigation

Column chromatographic fractionation of the *n*-hexane soluble fraction of the crude ethanol extract of leaves of *S. polygamus* led to the isolation of three compounds (I to III), whereas, the ethyl acetate soluble fraction afforded five compounds (IV to VIII) which were characterized through their physicochemical and spectral data.

Compound I

Lupeol acetate (0.04 g, crystallize from methanol as white needle crystals, m.p. (218°C), was suggested to be a triterpenoid since it responded positively to libermann-Burchard's test. It gives characteristic violet color with ρ-anisaldehyde/H_2SO_4 spray reagent. The $IR\sqrt{}_{Max}^{KBR}$ Spectrum, incorporated absorption bands at 1735 cm^{-1} which is characteristic for ester carbonyl group and exomethylene group at 1638 and 877 cm^{-1} (C=CH$_2$), 2924 and 2854 cm^{-1} which is characteristic for (C-H) stretching, 1459 cm^{-1}(C=C), 1246 cm^{-1} (C-O) and bending at 812. The EI/Mass (70 eV) m/z[M$^+$] at 468 m/z calculated for the molecular formula $C_{32}H_{52}O_2$. In addition to, the characteristic fragmentation peaks at 453 [M$^+$-CH$_3$] and 408 [M$^+$-CH$_3$COO] suggesting the presence of acetyl group in the structure.

The presence of m/z 249 ion indicates the presence of CH$_3$CO at C-3 position. Skeletal mass peaks are represented by m/z 218 and 203 and the base peak at 189 all in accordance with lupene skeleton of triterpenoid. The ^{1}HNMR spectrum (300 MHz in CDCl$_3$) exhibited four tertiary methyl signals of the lupene skeleton at 0.80, 0.86, 0.95, and 1.05 as singlets. The C-30 vinylic methyl at δ1.75 ppm (s) acetyl methyl resonate at δ1.97 ppm (s), where α-proton at C-3 appear at δ4.22 ppm dd (J=3, 6 Hz). The C-29 olefinic protons H$_a$, H$_b$ appeared at δ5.71 and 5.60 ppm as broad singlets. ^{13}CNMR (75 MHz, in CDCl$_3$ chemical shift assignments for compound I is revealed the presence of oxycarbon signal at δ83.0 assigned to (C-3) with downfield shift about (+4.0 ppm) compared with that of lupeol, which confirm the substitution with acetyl group (Chatterjee et al., 2000). Two signals assigned for olefinic carbons (C-20 and C-29) at δ=145.09 and 109.3 ppm, respectively and showed the presence of a carbonyl at δ172.8. All the aforementioned resonances are in accordance with compound I proposed structure and comparable to lupeol acetate (Jamal et al., 2008). As far as the available literature is concerned, this is the first report on isolation of lupeol acetate ($C_{32}H_{52}O_2$) from *S. polygamus*.

Compound II

Beta-sitosterol white powder (0.03 g); m.p.:134 to 135°C;

MS (m/z): exhibited molecular ion 414 (M$^+$), and other fragmentation pattern as 396, 339, 325, 310, 298, 257, 140, 139, 125, 97, 71, and 57. Its mass spectral data suggested the molecular formula as $C_{29}H_{50}O$. Compound II also showed positive Liebermann-Burchard reaction indicated its sterol nature. The ^{1}HNMR spectra (CDCl$_3$, 300 MHz) showed the presence of six methyl signals that appeared as two methyl singlet at δ0.68 and 1.01; three methyl doublets that appeared as δ0.81, 0.83, and 0.93; and a methyl triplet at δ0.84. Olefinic proton was presented at δ5.37 suggested the presence of tri substituted double bond at C-5/C-6 in its structure. The ^{1}HNMR spectra showed a proton corresponding to the proton connected to the C-3 hydroxy group which appeared as a triplet of doublet of doublets at δ3.53. The ^{13}CNMR showed twenty nine carbon signal including six methyls, eleven methylenes,ten methane together with three quaternary carbons. Thus, the structure of II was assigned as *β*-sitosterol that was consistent to the reported literature values (Jamal et al., 2008).

Compound III

Lupeol (0.25 g, crystallize from methanol as white needle crystals, m.p. (210 to 212), positive test for triterpenoid skeleton R$_f$ values, 0.76 in *n*-hexane: ethylacetate (6:4 v/v) and 0.35 *n*-hexane: chloroform (7:3 v/v); violet color with *p*-anisaldehyde/H_2SO_4 spray reagent. $IR\sqrt{}_{Max}^{KBR}$ Spectrum: incorporated absorption bands at 3415 cm^{-1} (OH), 2948, 2867 cm^{-1} (CH) and 1640, 885 cm^{-1} for (C=CH$_2$). EI Mass (70 eV) m/z showed a molecular ion peak (M$^+$) at 426.69 calculated for $C_{30}H_{50}O$ with characteristic fragment ions at 411 (M$^+$-Me), 393 (M$^+$-Me-H2O), 365, 299, 297, 245, fragment ions at m/z 220 and 207 (allocate the hydroxyl group at C$_3$ position), m/z 218, 205 and 189 all in accordance with lupene skeleton. ^{1}HNMR (300 Hz CDCl$_3$): revealed signals for seven tertiary methyl groups at 0.81 (3H,*s*,C$_{28}$-CH$_3$), 0.86 (3H,*s*,C$_{23}$-CH$_3$), 0.87 (3H,*s*,C$_{24}$-CH$_3$), 0.88 (3H,*s*,C$_{25}$-CH$_3$), 0.96 (3H,*s*,C$_{27}$-CH$_3$), 1.05 (3H,*s*,C$_{26}$-CH$_3$) and 1.70 (3H,*s*,vinylic methyl, C$_{30}$-CH$_3$), 3.14 (1H,*dd*,J=6 Hz, 10.5 Hz indicative of C$_3$-H is α- oriented) and 4.67 (1H,*s*,C$_{29}$-H$_b$), 4.55 (1H,*s*,C$_{29}$-H$_a$).

Compound IV

Gallic acid (0.50 g), brownish white powder crystallized from water as colorless needles m.p. (248 to 250°C). R$_f$ values (0.80 in *n*-butanol: acetic acid: water (4: 1: 5) v/v/v). In visible light deep brown color with NH$_3$. Violet fluorescence in UV and deep blue color with FeCl$_3$. UV λ$_{max}$ 274 nm in MeOH. $IR\sqrt{}_{Max}^{KBR}$ Spectrum: shows absorption peaks at 3238 cm^{-1} (OH), 1683 cm^{-1}(C=O), and 1613 cm^{-1} for C=C aromatic, and at 719, 771, 869 cm^{-1} (-C-H) aromatic. EI Mass (70 eV) m/z; [M$^+$] 170, 153 (M$^+$-H$_2$O), 125 [M$^+$-H-CO$_2$],79. ^{1}HNMR (300 Hz CD$_3$OD):

showed signals at 6.98 (2H, s, H-2, H-6).

Compound V

Methylgallate (0.08 g grayish white powder crystallized from absolute methanol give rise to colorless needles m.p. (199 to 203°C). R_f values (0.78 in *n*-butanol: acetic acid: water (4: 1: 5 v/v/v) shiny violet fluorescence in UV, deep brown color with NH3 in visible light, and dark blue color with FeCl$_3$ reagent.IR $\sqrt{}_{Max}$ ^{KBR} Spectrum: shows absorption peaks at 3521, 3367 cm^{-1} (OH), 1700 cm^{-1} for ester carbonyl group (C=O),1617 cm^{-1} and at 867, 758 cm^{-1} (-C-H) aromatic. UVλ_{max} 220 and 275 nm in MeOH. EI Mass (70 eV) m/z; [M$^+$]=m/z 184, 153 (M$^+$-OCH$_3$), [M$^+$-CO$_2$Me] =125 ^{1}HNMR (270 Hz CD$_3$OD): showed signals at 6.95 (2H,s,H-2,H-6) and 3.58 (3H,s,OMe).

Compound VI

Quercitrin (0.03 g, yellow powder needle crystallize from ethanol: water m.p. (182-184°C). 0.71 in S6=Ethylacetate: methanol: formic acid: water (80: 20: 5: 1 v/v/v/v), Visible Yellow. UV: dark purple. UV/NH$_3$.Yellow. AlCl$_3$; Yellow. UVλ_{max} nm: MeOH:257,297sh,352 (flavonol) NaOMe: 270, 355, 405n nm (free OH on ring A&B). AlCl$_3$:268, 333, 430 (free OH on ring A&B) AlCl$_3$/HCl: 272, 353, 405 (free OH at C-5). NaOAc: 267, 322 sh, 372 (free OH at C-5&ring B) NaOAc/H$_3$BO$_3$: 260,300sh, 367 (*ortho* groups at ring B. ^{1}HNMR spectrum showed, Aglycone: 7.34 (1H,d, J=2.1 Hz, H-2`), 7.32 (1H,dd, J=2.1, 8.4 Hz, H-6`), 6.92 (1H,d, J=8.4 Hz, H-5`), 6.37 (1H,d, J=2.1 Hz, H-8), 6.20 (1H, d, J=2.1 Hz, H-6). Sugar: 5.35 (1H,d, J=1.2 Hz, H-1``), 4.2 (1H,dd, J=1.5, 3.4 Hz, H-2``), 3.71 (1H,dd, J=3.4, 8.9 Hz, H-3``), 3.47-3.32 (m, H-4``,5``) and 0.95 (3H,d, J= 6 Hz, CH$_3$-rhamnose, $^{13}$CNMR spectrum showed: 177.43 (C-4), 166.1 (C-7), 160.08 (C-5), 158.38 (C-9), 158.18 (C-2), 149.8 (C-4`), 143.85 (C-3`), 136.0 (C-3), 123.13 (C-1`), 123.5 (C-6`), 116.25 (C-5`), 117.26 (C-2`), 104.3 (C-10), 102.54 (C-1``), 96.1 (C-6), 94.7 (C-8), 72.16 (C-5``), 72.04 (C-3``), 71.94 (C-2``), 73.28 (C-4``), 17.65 (C-6``).

Compound VII

Kaempferol (0.03 g, yellow powder soluble in methanol m.p. (276 to 278°C). R_f value 0.88 in S6: Ethylacetate: methanol: formic acid: water (80:20:5:1 v/v/v/v) color of spot in visible pale yellow. UV: dark yellow, NH$_3$/UV: dark yellow. AlCl$_3$/visible: bright yellow. AlCl$_3$/UV: fluorescent green. UVλ_{max} nm MeOH: 268, 329sh, 369 (flavonol). NaOMe: 278, 326sh, 416 (free OH on ring A&B). AlCl$_3$: 268, 304sh, 345, 424 (free OH on ring A&B). AlCl$_3$/HCl: 269, 303sh, 350, 424 (free OH at C-5&no *ortho* OH at ring B). NaOAc: 275, 300 sh, 387 (free OH at C-7). NaOAc/H$_3$BO$_3$:267, 295sh, 373 (no *ortho* OH groups at ring B). ^{1}HNMR (300 MHz CD3OD), 8.29 (2H,d, J=9Hz, H-2`,H-6`), 7.11 (2H,d, J=8.7 Hz, H-3`,H-5`), 6.59 (1H,d, J=1.8 Hz, H-8), 6.38 (1H,d, J= 1.8 Hz, H-6).

Compound VIII

Quercetin (0.05 g, yellow powder needle crystallize from ethanol: waterm.p. (182-184°C). R_f value 7.0 in S6 = Ethyl acetate: methanol: formic acid: water (80:20:5:1 v/v/v/v) color of spot in Visible: Yellow. UV: dark purple. UV/NH$_3$: Yellow. AlCl$_3$/visible: bright yellow. AlCl$_3$/UV: Yellow. UVλ_{max}nm MeOH: 255, 301sh, 368 (flavonol). NaOMe: 246, 406 (free OH on rings A&B). AlCl$_3$: 269, 457 (free OH on rings A&B). AlCl$_3$/HCl: 266, 303sh, 353, 430 (free OH at C-3, C-5 & *ortho* OH at ring B). NaOAc: 271, 329 sh, 390 (free OH at C-7 & ring B). NaOAc/H$_3$BO$_3$: 260, 384 (*ortho* OH groups at ring B). ^{1}HNMR (300 MHz CD$_3$OD), 7.91 (2H,d, J= 2.1 Hz, H-2`), 7.82 (1H,dd, J=2.1, 9 Hz, H-6`) 7.07 (1H,d, J= 8.4 Hz, H-5`), 6.57 (1H,d,J= 2.1 Hz, H-8), 6.37 (1H,d, J= 1.8 Hz, H-6).

The isolated compounds I to VIII were identified by comparison of MS, IR, ^{1}H-NMR and ^{13}C-NMR data to previously reported ones and were identified as lupeol acetate (I) (Ogunkoya, 1981) β-sitosterol (II) (Saha et al., 2011; Goad and Akihisa, 1997) and lupeol (III) (Dantanrayana et al., 1982), gallic acid (IV), methyl gallate (V), quercetin (VI), kaempferol (VII, quercetin-3-*α*-O-rhamnoside (VIII) (Hussein et al., 2003; Gutzeit et al., 2007; Marzouk 2008). It is noteworthy to mention that this is the first report of compounds I (lupeol acetate), III (lupeol) and VIII (quercetin-3-*α*-O-rhamnoside) from the leaves of *S. polygamus* growing in Egypt. Compounds III (lupeol) and IV (gallic acid) were detected as major compounds from the leaves of the plant so the biological activity, hepatoprotection and antioxidant effects against toxicity induced by CCL$_4$ was carried out on them. Shabana et al. (2013) reported that the crude ethanol extract of leaves at dose 100 mgkg^{-1}exhibited a significant hepatoprotective activity. Therefore, this study was undertaken to investigate the phytochemicals to which these activity could be attributed.

In vivo hepatoprotective activity

The control group (received saline) served as a baseline for all the biochemical parameters. A significant increase in the activity of the serum enzymes AST, ALT and ALP ($P<0.05$) was observed upon treatment with CCl$_4$. Positive control (Silymarin group) showed highly significant decrease ($P<0.05$) in AST, ALT and ALP as compared to negative control group. The treatment of intoxicated rats with compounds III and IV at a dose of (50 and 100 mg kg^{-1}) produced a significant hepatoprotective effects. Hepatic protection was evidenced by the ability of gallic acid to normalize the

Table 1. Effects of the of the isolated phytochemicals of *S. polygamus* *and silymarin** drug in male albino rats(n=6)on serum AST, ALT, and ALP levels.

Group		Zero time Mean±SE	7 days[a] Mean±SE	72 h[b] Mean±SE	72 h[b] % Change[c]	7 days[b] Mean±SE	7 days[b] % Change[d]
AST (u/L)	Control	36.8 ± 1.1	36.3 ± 1.5	159.7 ± 4.9*	333.9	161.6±6.2*	1.1
	L (50 mg/kg b. wt.)	37.9± 1.3	37.6±1.4	116.2±4.3*	206.5	86.4±2.7*•	25.6
	L (100mg/kg b. wt.)	39.4±1.6	39.1±1.5	81.7±2.1*	107.3	63.5±2.4*•	22.2
	G (50 mg/kg b. wt.)	39.1±1.2	38.7±1.3	91.5±3.7*	134.0	79.5±2.8*•	13.1
	G (100mg/kg b. wt.)	38.1±1.4	37.8±1.2	68.5±2.4*	79.7	49.2±3.1*•	28.1
	Silymarin**	38.4 ± 1.4	37.8 ± 1.2	51.2 ± 2.6*	33.3	37.2 ± 1.4*•	27.3
ALT (u/L)	Control	37.8 ± 7.4	37.3 ± 1.5	166.1 ± 7.8*	339.4	167.2 ± 6.8*•	0.6
	L (50 mg/kg b. wt.)	40.9±1.5	40.6±1.6	103.5±4.8*	153.0	98.5±3.2*•	4.8
	L (100mg/kg b. wt.)	43.6±1.7	43.2±1.4	78.4±2.6*	79.8	61.4±3.2*•	21.6
	G (50 mg/kg b. wt.)	36.2±1.2	35.8±1.1	89.8±2.5*	148.0	68.6±2.1*•	23.6
	G (100mg/kg b. wt.)	39.7±1.6	39.1±1.5	61.6±3.1*	55.1	53.3±2.1*•	13.4
	Silymarin**	41.2 ± 1.7	40.8 ± 1.8	53.7 ± 1.8*	30.3	38.9 ± 1.4*•	27.5
ALP (KAU)	Control	7.4 ± 0.1	7.4 ± 0.1	55.3 ± 3.4*	647.2	68.2 ± 3.8*•	23.3
	L (50 mg/kg b. wt.)	7.5±0.1	7.6±0.1	41.6±0.3*	454.6	37.2±1.7*•	10.5
	L (100mg/kg b. wt.)	7.7±0.1	7.4±0.1	33.5±0.7*	335.0	24.3±0.8*•	27.4
	G (50 mg/kg b. wt.)	7.6±0.1	7.4±0.1	39.2±0.3*	415.7	28.3±0.8*•	27.3
	G (100mg/kg b. wt.)	7.8±0.1	7.7±0.1	26.1±0.7*	234.6	18.7±0.3*•	28.3
	Silymarin**	7.3 ± 0.1	7.0 ± 0.1	18.9 ± 0.6*	158.9	7.4 ± 0.1•	60.8

*Statistically significant different from zero time group at $p < 0.01$. •Statistically significant different from 72h group at $p < 0.01$. [a]Pretreated with tested sample. [b]After induction of liver damage. *Phytochemicals, L=lupeol; G=gallic acid were tested at (100, 50 mg/kg b wt). **Silymarinat dose level (25 mg/kg b wt). [c]%change (change in liver enzymes after induction of liver disease from zero time). [b]%change (change in liver enzymes after 7 days from induction of liver disease).

high enzyme parameters in a dose-dependent manner (50 and 100 mgkg[-1]) by 47.9 and 102.9% for AST comparable to silymarin. Gallic acid (100 mgkg[-1]) exhibited a highly significant reduction ($p<0.05$) in AST, ALT and ALP better than lupeol. Results presented in Table 1 showed that low dose, 50 mgkg[-1] of lupeol exhibited normalization by 93%, for AST comparable to silymarin.

In vivo antioxidant activity

Concerning the antioxidant activity, the phytochemicals of the leaves of *S. polygamus* exhibited significance antioxidant activity. Results (Table 2) revealed that lupeol and gallic acid in dose 100 mgkg[-1] exerted a remarkable antioxidant activity (91.4 and 96.4%), respectively. The reduced level of glutathione in intoxicated rats was greatly restored by lupeol and gallicacid relative to vitamin E (potency 100%). These results clearly elucidated the significant *in vivo* antioxidant activity provided by both lupeol and gallic acid, so they could be considered as powerful antioxidant agents. Gallic acid showed highly significant rise ($p<0.05$) in glutathione

better than lupeol.

DISCUSSION

Free radicals and reactive oxygen species (ROS) play a role in liver diseases pathology and progression, dietary antioxidants have been proposed as therapeutic agents to counteract liver damage (Higuchi and Gores, 2003). Natural antioxidant may act as protectors but, more importantly, may exert modulator effects in cells through actions in antioxidant, drug metabolizing and repairing enzymes as well as working as signaling molecules in important cascades for cell survival (Singh et al., 2008).

Terpenoids are useful phytochemicals isolated from plants (Firn, 2010). They can regulate the activity of nuclear hormone receptors, which are involved in maintain the energy homeostasis in the body and manage obesity induced metabolic disorders including type 2 diabetes, hyperlipidemia, insulin resistance and cardiovascular disease (Goto et al., 2010).

In the present study, the *in vivo* antioxidant and hepatoprotective activities of major isolated compounds (lupeol, gallic acid) from the leaves of *S. polygamus* on

Table 2. Antioxidant activity of the isolated phytochemicals of *S. polygamus* in male albino rats (n=6).

Group	Blood glutathione (mg%)	Change (%)[b]	Potency (%)[c]
Control (1 ml saline)	36.6±1.3	-	-
CCl$_4$ non treated	22.8±0.5*	37.70	-
CCl$_4$ treated with lupeol (100 mg/kg)	33.1±1.4*	9.56	91.43
CCl$_4$ treated with gallic acid (100 mg/kg)	35.3±1.3*	3.55	96.44
CCl$_4$ treated with Vitamin E (7.5 mg/kg)	36.2±1.2*	1.09	100

*Statistically significant different from control group at $p < 0.01$. [b]% of change from control= $(M_c - M_t) \times 100 / M_c$; M_c is the mean change in control animals; M_t is the mean change in drug-treated animals; [c]% Potency calculated as regard the standard drug.

experimental rats were evaluated. The hepatoprotection showed that the tendency of these molecules to preserve liver enzymes was particularly pronounced with gallic acid. The results of the present study are in accordance with the previously reported reviews, which demonstrated that lupeol ameliorates aflatoxin B1-induced peroxidative hepatic damage in rats (Preetha et al., 2006). Hepatoprotective effect of lupeol on tissue defense system in cadmium-induced hepatotoxicity in rats was known (Sunitha et al., 2001). In relation to that, it was observed that lupeol can substantially normalized degenerative alterations in the hepatocytes with granular cytoplasm (Akçam et al., 2013). Additionally, lupeol induced growth inhibition and apoptosis in hepatocellular carcinoma SMMC7721 cells by down-regulation of the death receptor expression (Zhang et al., 2009). Moreover, have some effect on lipid peroxidation and antioxidant status in rat kidney after chronic cadmium exposure (Nagaraj et al., 2000).

Henceforth, Lupeol has several biological activities, anti-inflammatory, and anti-tumor activities wherein according to some review (Wal et al., 2011; Gallo and Sarachine, 2009; Ragasa et al., 2009), was previously identified in family Anacardiaceae (Shabana et al., 2011). Lupeol and its ester derivative, lupeollinoleate were investigated for their possible hepatoprotective effect against cadmium-induced toxicity in rats (Sunitha et al., 2001). The mechanism of cadmium-mediated acute hepatotoxicity involves two pathways, one for the initial injury produced by direct effects of cadmium and the other for the subsequent injury produced by inflammation. Primary injury appears to be caused by the binding of Cd^{2+} to sulfhydryl groups on critical molecules in mitochondria. Thiol group inactivation causes oxidative stress, the mitochondrial permeability transition, and mitochondrial dysfunction (Rikans and Yamano, 2000). The fact that antioxidant agents inhibit carbon tetrachloride-induced liver damage (Wu et al., 2009) prompt to study the antioxidant effect of the isolated compounds.

Phenolics have been known to possess a capacity to scavenge free radicals (Valenzuela et al., 2003).Gallic acid, phenolic acid possesses antioxidant and anticancer activities (You et al., 2010; Ji et al., 2006; Chanwitheesuk

et al., 2007).

Gallic acid with the lowest hydrophobicity than methylgallate and *alpha*-tocopherol was found to be the most active antiradical agent (Asnaashari et al., 2014). The low hydrophobicity of gallic acid can be treated by using gallic acid–lecithin complex (Liu et al., 2014). The hepatoprotective and antioxidant effects of gallic acid were evaluated against paracetamol-induced hepatotoxicity in mice (Rasool et al., 2010). Gallic acid showed significant protection against paracetamol induced liver injury in rats by improving the antioxidant defense condition, lessening lipid peroxidation and conserving the pathological changes of the liver. Hepatotoxicity, results not from paracetamol itself, but from one of its metabolites *N*-acetyl-P-benzoquinoneimine (NAPQI), which depletes the liver's natural antioxidant glutathione and directly damages cells in the liver, leading to liver failure (Rasool et al., 2010).

Carbon tetrachloride is a well-known hepatotoxic agent and the preventive action of drugs on liver damage by CCl$_4$ has been widely used as an indicator of their liver protective activity. Changes associated with CCl$_4$-induced liver damage are similar to that of acute viral hepatitis (Huang et al., 2010). Carbontertachloride treatment initiated lipid peroxidation, caused leakage of enzymes like alanine tranaminase and lactate dehydrogenase (Singh et al., 2008; Krithika et al., 2009) levels. CCl4-induced lipid peroxidation as well as covalent binding of CCl$_4$ metabolites to cell components, and also restored lipoprotein metabolism (Boll et al., 2001). The anti-oxidant vitamin E (alpha-tocopherol) blocked lipid peroxidation, but not covalent binding, and secretion of lipoproteins remained inhibited. The covalent binding of the CCl^{3*} radical to cell components initiates the inhibition of lipoprotein secretion and thus steatosis, whereas reaction with oxygen, to form CCl^{3*}OO*, initiates lipid peroxidation. The former process may result in adduct formation and, ultimately, cancer initiation, whereas the latter results in loss of calcium homeostasis and, ultimately, apoptosis and cell death (Boll et al., 2001).

In CCl$_4$-induced liver injury model, oxidative stress could be provoked, which prompts lipid peroxidation that injure hepatocellular membrane, followed by substantial

release of pro-inflammatory chemokines and cytokines, which in consequence of liver damage (Feng et al., 2011). A large amount of plants, especially medicinal plants, has been investigated to eliminate the hepatic damage stimulated by CCl$_4$. For example, *Coptidis rhizome*, a traditional Chinese medicinal plant used to clear heat and scavenge toxins, belongs to liver meridian in Chinese medicinal practice (Feng et al., 2010, 2012).

Anti-oxidative therapy, mainly using natural antioxidants, represents a reasonable therapeutic approach for the prevention and/or treatment of liver diseases due to the role of oxidative stress in initiation and progression of hepatic damage (Li et al., 2015). However, although concept of anti-oxidative therapy has been improved nowadays, there is a long way to go for the application of antioxidants in liver disease. Therefore, translational research is of great importance for anti-oxidative therapy. Considering ROS and oxidative stress act positively in certain circumstances and the difference between animals and humans, the effective dose and safe dose, duration of treatment, absorption and bio-availability of antioxidants require thorough investigation. Furthermore, in the future, large-scale samples and appropriate duration of anti-oxidative treatment for liver diseases should be performed (Li et al., 2015).

Conclusion

The current study provides a scientific basis for the claims that *S. polygamus* is effective against certain liver–related diseases (Shabana et al., 2013). Taken together, our findings provide evidence that the major isolated compounds (lupeol and gallic acid) from *S. polygamus* exhibit hepatoprotective and antioxidant activities. These compounds might be useful for the prevention of toxic-induced and free radical-mediated liver diseases since it has been suggested that antioxidant compounds may be used as prophylactic agent.

Moreover, this study demonstrates that *S. polygamus* have potential hepatoprotective activity which is mainly attributed to the antioxidant potential, which might occur by reduction of lipid peroxidation and cellular damage. Previous reports were prevailed the isolation of lupeol and gallic acid from different genera belongs to Family Anacardiaceae (Shabana et al., 2008, 2011). As a part of the ongoing investigation of plants belongs to family Anacardiaecae (Shabana et al., 2008, 2011, 2013), it can be concluded that gallic acid and lupeol could be consider as potent hepatoprotective agents and chemotaxonomic markers for plants which belongs to family Anacardiaecae.

Conflict of interest

The authors have not declared any conflict of interests.

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