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Research Article

Chemical Constituents and Antioxidant Activity of a Polar Extract from *Pituranthos battandieri* Maire

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ABSTRACT

Phytochemical investigation of the *n*-butanol soluble part of the aqueous-MeOH extract of the aerial parts of *Pituranthos battandieri* Maire collected from the region of Bechar in the south-west of Algeria, led to the isolation and structural elucidation of mannitol **1**, xanthotoxol **2**, 8-(6',7'-dihydroxygeranyloxy)-psoralen **3** and isorhamnetin 3-*O*-ß-D-glucopyranoside (cacticin) **4**. The structures were established by spectral analyses, mainly ESI-HRMS, UV and NMR experiments (^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC) and comparison with literature data. All these compounds were described for the first time from this endemic species. The free radical scavenging activity of this extract was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) and hydrogen peroxide (H_2O_2) scavenging activity models. Antihemolytic property was performed using H_2O_2 induced red blood cell hemolysis model. Antiperoxidative effect was evaluated using lipid peroxidation-ammonium thiocyanate model. Both DPPH and H_2O_2 assays indicated antioxidant ability of *n*-BuOH extract of *P. battandieri* (BEPB), that were reflected by IC_{50} values 876.16±7.96 µg/mL and 603.8±71.06 µg/mL, respectively. In addition, the results of the present study clearly indicated that BEPB inhibited rat erythrocytes hemolysis induced by H_2O_2 in a dose dependent way with IC_{50} value 782.38±89.97 µg/mL. The inhibition of lipid peroxides generated from linoleic acid was recorded at IC_{50}: 496.04±117.06 µg/mL. The antiperoxidative and the antihemolytic activities of *P. battandieri* appear as a consequence of synergistic interactions between active constituents among them mannitol **1**, the furanocoumarins **2** and **3**, and the flavonoid **4**.

Keywords: *Pituranthos battandieri* Maire, *Deverra battandieri* (Maire) Chrtek, Apiaceae, Antiperoxidative, Antihemolytic, Antioxidant.

INTRODUCTION

Reactive oxygen species (ROS) that were continuously produced in human cells could induced oxidative stress initiating cell death by macromolecules damage[1,2]. The polyunsaturated fatty acids are the most susceptible to the ROS, leading to the lipid peroxidation which is probably a crucial step in the pathogenesis of several diseases states[3,4]. Lipid peroxidation could lead to the loss of structure and function of membranes and generation of cytotoxic and mutagenic end products which can spread the oxidative reactions and cause further damage[5,6]. Red blood cells (RBCs) are considered as prime targets for free radical attack owing to the presence of high membrane concentration of polyunsaturated fatty acids, the oxidative stress on RBC is implicated in hemolysis which occurs in a variety of pathological conditions[7,8]. Hemolysis which refers to destruction of erythrocytes with liberation of hemoglobin in the plasma, occurs in a variety of pathological conditions[9]. A number of plants such as *Hibiscus esculentus*[10], *Allium paradoxum*[11], *Ugni molinae* Turcz[12], *Wrightia tinctoria*[13] showed anti-anemic properties due to the presence of a variety of flavonoids. Natural antioxidant compounds like flavonoids and coumarins have been proposed as an effective approach for the prevention and treatment of multiple RBC disorders *via* scavenging free radicals or lipid peroxyl inhibiting effect[14-16]. Focusing our attention on natural sources of antioxidants and taking in the consideration that many plants from Apiaceae family have been used in traditional medicine to treat various diseases, including asthma, gastrointestinal disorders, intestinal parasites, and have been known to possess antifungal, anti-diabetic, anti-inflammatory, antimutagenic and antiviral activities[17], we investigated a species of this family from the genus

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Pituranthos. From the chemical composition viewpoint, the members of this family are well known producers of coumarins and furanocoumarins[18] which are known to exhibit valuable biological properties such as analgesic, anti-inflammatory, antibacterial, antiviral, antiproliferative, in addition to their well known photosensitizing effect[19-25]. The genus *Pituranthos* includes more than 20 species[26], some of them are used in traditional medicine for the treatment of asthma, rheumatism, digestive difficulties, urinary infections, and scorpions stings[27-29]. As a consequence, several *Pituranthos* species were investigated in terms of biological activities[30-34]. Most of the species of this genus are observed in North Africa. In Algeria, the genus *Pituranthos* Viv. (*Deverra* DC.*)* is represented by four species from which *Pituranthos battandieri* Maire, synonym: *Deverra battandieri* (Maire) Chrtek. This plant is a rare endemic of Saharan areas of Morocco and Algeria[35,36]. In Algeria it grows in the South of the country, especially in the area of Bechar. According to our previous studies on Saharan species which showed the presence of high content of bioactive compounds and positive antioxidant, anti-inflammatory and antiproliferative properties[37-41], we investigated this species on which we previously reported the chemical composition of the essential oil of its aerial parts[42]. In this paper we report for the first time, the chemical constituents of the *n*-butanol soluble part of the aqueous-MeOH extract (BEPB) of its aerial parts. BEPB was also investigated for antiperoxidative damage and antihemolytic properties using different *in vitro* model assays.

MATERIALS AND METHODS

Phytochemical studies

General Procedures

TLC: pre-coated aluminium foil silica gel $60F_{254}$ (Merck). Column chromatography (CC): silica gel 60 (Merck 230-400 mesh). UV Spectra (MeOH): Shimadzu (190-3200 nm, UV-3101PC) spectrophotometer. NMR spectra: Bruker AMX-400 MHz, AMX-500 MHz and Avance DPX-250, 250 MHz spectrometers; chemical shifts (δ) are given in ppm using TMS as internal standard and coupling constants (*J*) are given in Hz. High resolution mass spectra (ESI-HRMS) were performed on a Agilent 6520 Accurate Mass Q-TOF (Agilent Corporation, Santa Clara, CA, USA) and a μ-QTOF spectrometer (Bruker Daltonics, Wissembourg, France).

Plant material

The aerial parts of *Pituranthos battandieri* Maire, was collected from Bechar Southern Algeria in April 2010. The plant material was authenticated by M. Mohamed Benabdelhakem, director of the nature preservation agency, Bechar. A voucher specimen (PBA 54/04/10) was deposited at the Herbarium of the VARENBIOMOL Research unit, University Frères Mentouri Constantine 1.

Extraction and Isolation

Air-dried Aerial parts of *Pituranthos battandieri* Maire (3350 g) were macerated at room temperature with MeOH-H_2O (80:20, *v/v*) for 48 h, three times. After filtration, the filtrate was concentrated (1100 mL) and dissolved in H_2O (1300 mL). The resulting solution was extracted successively with petroleum ether, chloroform, ethyl acetate and *n*-butanol. The organic layers were dried with Na_2SO_4, filtered and concentrated in vacuum up to 35°C to obtain the following extracts: petroleum ether (0.9 g), $CHCl_3$ (17 g), EtOAc (12 g) and *n*-BuOH (70 g), respectively. During the concentration of the *n*-BuOH extract, a white precipitate was formed. This precipitate was filtered and washed with a solution of $CHCl_3$ and a little amount of MeOH to yield mannitol **1** (2.5 g)[43]. Part of the *n*-BuOH extract (20 g) was fractionated by column chromatography (230-400 mesh silica gel; $CHCl_3$/MeOH with increasing polarity) to give 28 fractions (F_1-F_{28}) obtained by combining the eluates on the basis of TLC analysis. Fraction F_5 (66.4 mg) ($CHCl_3$/MeOH; 99:1) was submitted to preparative plates of silica gel 60, HF_{254} ($CHCl_3$/Acetone; 9:1, two elutions) to give xanthotoxol **2** (20.6 mg)[44]. Fraction F_{11} (45.1 mg) ($CHCl_3$/MeOH; 98:2) was submitted to preparative plates of silica gel ($CHCl_3$/Acetone; 9:1, two elutions) and purified over Sephadex LH-20 column eluted with MeOH to afford 8-(6',7'-dihydroxygeranyloxy)-psoralen **3** (11.0 mg)[45,46]. Fraction F_{21} (1254.9 mg) ($CHCl_3$/MeOH; 90:10) was rechromatographed on a silica gel column (AcOEt/MeOH/H_2O; 8:1:1) to yield 3 sub-fractions ($subF_1$-$subF_3$). $SubF_1$ (25.2 mg) was submitted to preparative plates of silica gel ($CHCl_3$/MeOH; 6:1) to give a compound which was purified on a Sephadex LH-20 column eluted with methanol, to obtain isorhamnetin 3-*O*-β-glucopyranoside (cacticin) **4** (18.0 mg)[47].

The structures of the isolated compounds were elucidated by UV, HR-ESIMS, ^1H and ^{13}C NMR and 2D NMR experiments (COSY, NOESY, HSQC and HMBC) (Figure 1). All these results were in good agreement with the literature data.

Biological studies

DPPH• scavenging activity

The antioxidant activity was evaluated by monitoring its ability in quenching the stable free radical DPPH[48]. Different methanol dilutions of BEPB were mixed with 1 mL of DPPH• 0.2 mM methanol solution. After 30 minutes, the readings were made at 517 nm. A solution of DPPH• (1 mL, 0.2 mM) in methanol (1 mL) was used as a negative control, ascorbic acid and trolox were used as positive control. The percentage of DPPH• scavenging activity (I %) was calculated using the equation (1).

(1): $I\% = [(A_0 - A_1)/ A_0] \times 100$

Where A_0 is the absorbance of DPPH• solution alone, A_1 is the absorbance of BEPB / ascorbic acid / trolox.

Hydrogen peroxide (H_2O_2) scavenging activity

The H_2O_2 scavenging ability of the BEPB was examined according to Gulcin *et al.*, 2003[49] with minor modifications. To 3.4 mL of BEPB (100-1200 μg/mL) in phosphate buffer 50 mM (pH 7.4), 0.6 mL of H_2O_2 was added and incubated at room temperature for 10 min. Decrease in the absorbance of H_2O_2 upon oxidation was monitored at 230 nm spectrophotometrically against suitable blank (phosphate buffer alone). Trolox was used as a standard antioxidant and control (phosphate buffer and

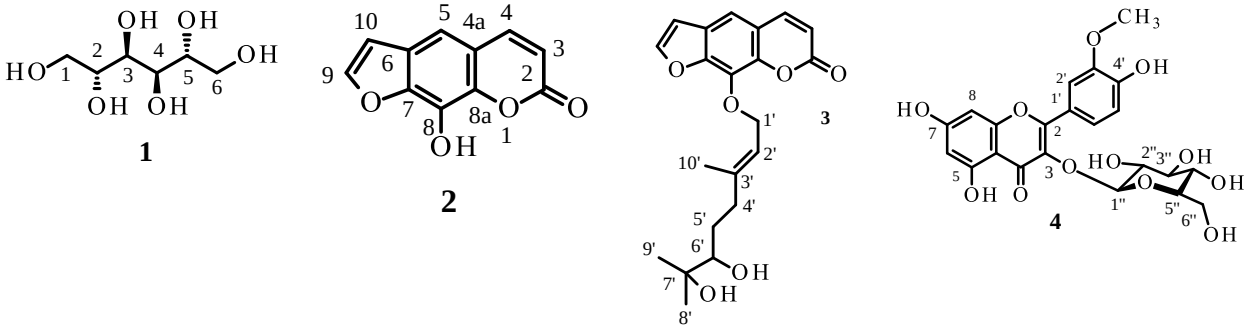


Figure 1: Structures of compounds **1 – 4.**

H_2O_2) was prepared and the percentage inhibition was calculated using the above equation (1).

Anti-erythrocyte hemolysis

The inhibitory activity of H_2O_2 induced red blood cell damage was evaluated by the method described by Ebrahimzadech *et al.*, 2009[10]. To 100 µL of 5% (*v*/*v*) suspensions of erythrocytes in phosphate buffer saline (PBS), 50 µL of BEPB (100-1200 µg) in PBS pH (7.4) was added; to this, 100 µL of 100 µM H_2O_2 was added. The reaction mixture was shaken gently while being incubated at 37°C for 3 hours. The reaction mixture was diluted with 8 mL of PBS and centrifuged at 2000 *x g* for 10 minutes. The absorbance of the resulting supernatant was measured at 540 nm by spectrophotometry to determine hemolysis. Likewise erythrocytes were treated with 100 µM H_2O_2 and without inhibitors to obtain complete hemolysis. The pourcentage of hemolysis inhibition was calculated using the equation (2).

(2): % hemolysis inhibition = $[(A_0 - A_1)/ A_0] \times 100$

Where A_0 is the absorbance of H_2O_2-erythrocytes, A_1 is the absorbance of H_2O_2-erythrocytes + BEPB / trolox.

Lipid peroxidation-ammonium thiocyanate.

The inhibition of lipid peroxidation by BEPB was estimated by the method of thiocyanate using linoleic acid as a source of peroxide[50]. The generated peroxide reacted with the ferrous chloride (Fe^{2+}) to form the ferric ion (Fe^{3+}) monitor form of a thiocyanate complex. The emulsion of linoleic acid was prepared by homogenization of linoleic with Tween-40 emulsified in phosphate buffer (0.2 M, pH 7). The samples were prepared in MeOH/water and the selected concentrations for the study were 500, 600 and 700 µg/mL. After incubation at 37°C, the ammonium thiocyanate and ferrous chloride were added. The mixture prepared by the same procedure was used as control. Trolox was used as reference. Powered the coloration was measured at 500 nm for 4 successive days. Results were calculated according to the equation (1).

Statistical Analysis

All the experiments were done in triplicates and the data expressed as mean±standard deviation (SD), $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Isolated and identified compounds

The isolated and identified compounds are reported in Figure 1.

*D-Mannitol (**1**)*: HRESI-MS (+): *m/z* 205.0689 [M+Na]+, calculated for $C_6H_{14}O_6Na$: 205.0688, formula: $C_6H_{14}O_6$; ^{1}H-NMR (400 MHz, DMSO-d_6), δ(ppm), *J*(Hz): 4.12 (2H, *d*, *J* = 6.9 Hz, 2OH), 4.31 (2H, *t*, *J* = 5.6 , 2OH), 4.40 (2H, *d*, *J* = 5.4 , 2OH), 3.61 (2H, *m*, H-1a, H-6a), 3.54 (2H, *t*, H-3, H-4), 3.45 (2H, *m*, H-2, H-5), 3.38 (2H, *m*, H-1b, H-6b); ^{13}C-NMR (DMSO-d_6, 100 MHz, δ ppm): 71.3 (C-2, C-5), 69.7 (C-3, C-4), 63.8 (C-1, C-6).

*Xanthotoxol (**2**):* HRESI-MS (+): *m/z* 225.0167 $[M+Na]^+$, corresponding to $C_{11}H_6O_4Na$ (calculated for $C_{11}H_6O_4Na$: 225.0164), Formula: $C_{11}H_6O_4$; ^{1}H-NMR (500 MHz, MeOH-d_4, δ ppm, *J*/Hz): 6.24 (1H, *d*, *J* = 9.6, H-3), 7.86 (1H, *d*, *J* = 9.6, H-4), 7.22 (1H, *s*, H-5), 7.69 (1H, *d*, *J* = 2.2, H-9), 6.77 (1H, *d*, *J* = 2.2, H-10); ^{13}C-NMR (MeOH-d_4, 125 MHz, δ ppm): 163.24 (C-2), 114.68 (C-3), 147.21 (C-4), 117.76 (C-4a), 111.32 (C-5), 127.36 (C-6), 147.15 (C-7), 131.74 (C-8), 141.02 (C-8a), 148.23 (C-9), 108.00 (C-10).

*8-(6',7'-dihydroxygeranyloxy)-psoralen (**3**):* HRESI-MS (+): accurate mass 372.15713 (calculated for $C_{21}H_{24}O_6$: 372.15729), formula: $C_{21}H_{24}O_6$; ^{1}H-NMR (400 MHz, DMSO-d_6, δ ppm, *J*/Hz): 6.42 (1H, *d*, *J* = 9.6, H-3), 8.14 (1H, *d*, *J* = 9.6, H-4), 7.68 (1H, *s*, H-5), 8.11 (1H, *d*, *J* = 2.4, H-9), 7.08 (1H, *d*, *J* = 2.4, H-10), 4.92 (2H, *d*, *J* = 6.8, H-1'), 5.50 (1H, *t*, *J* = 7.0, H-2'), 1.89 (1H, *m*, H-4'a), 2.17 (1H, *m*, H-4'b), 1.53 (1H, *m*, H-5'a), 1.10 (1H, *m*, H-5'b), 2.98 (1H, *d*, *J* = 10.4, H-6'), 0.95 (1H, *s*, H-8'), 1.00 (1H, *s*, H-9'), 1.60 (1H, *s*, H-10'); ^{13}C-NMR (DMSO-d_6, 100 MHz, δ ppm) : 166.10 (C-2), 114.27 (C-3), 145.31(C-4), 116.32 (C-4a), 118.81 (C-5), 127.36 (C-6), 147.95 (C-7), 130.42 (C-8), 143.41 (C-8a), 147.80 (C-9), 107.05 (C-10), 69.29 (C-1'), 118.81 (C-2'), 143.37 (C-3'), 36.44 (C-4'), 29.18 (C-5'), 76.90 (C-6'), 71.52 (C-7'), 24.56 (C-8'), 26.19 (C-9'), 16.28 (C-10').

*Cacticin (**4**):* $C_{22}H_{22}O_{12}$, UV (MeOH, λ_{max}, nm): 350, 253; +NaOH: 410 (hyperchromic effect), 325, 273; +$AlCl_3$: 403, 253; + $AlCl_3$/HCl: 400, 253; +NaOAc: 395, 273; + NaOAc/H_3BO_3: 351, 253; ^{1}H-NMR (250 MHz, MeOH-d_4, δ ppm, *J*/Hz): 7.95 (1H, *d*, *J* = 1.9 , H-2'), 7.61 (1H, *dd*, *J* = 8.5; 1.9, H-6'), 6.92 (1H, *d*, *J* = 8.5, H-5'), 6.30 (1H, *brs*, H-8), 6.10 (1H, *brs*, H-6), 5.30 (1H, *d*, *J* = 7.3, H-1''of glucose), 3.90 (3H, *s*, 3'-OCH_3).

DPPH• scavenging activity of BEPB

Our results revealed that BEPB exhibited a maximum (59.86-78.40%) DPPH•scavenging effect at the concentration ranging between 1000-1200 µg/mL. While the standard references, trolox and ascorbic acid needed

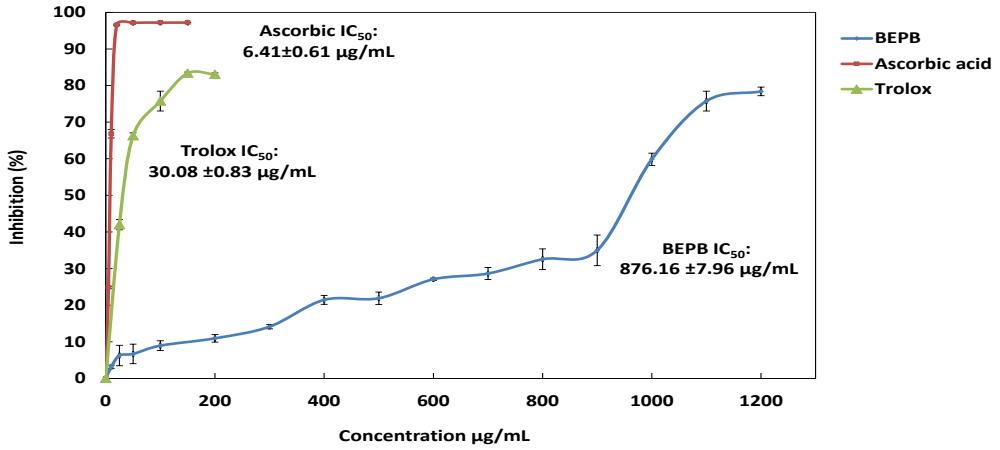


Figure 2: DPPH• scavenging activity of BEPB and standards. Values are means ± SD (n=3) P<0.05.

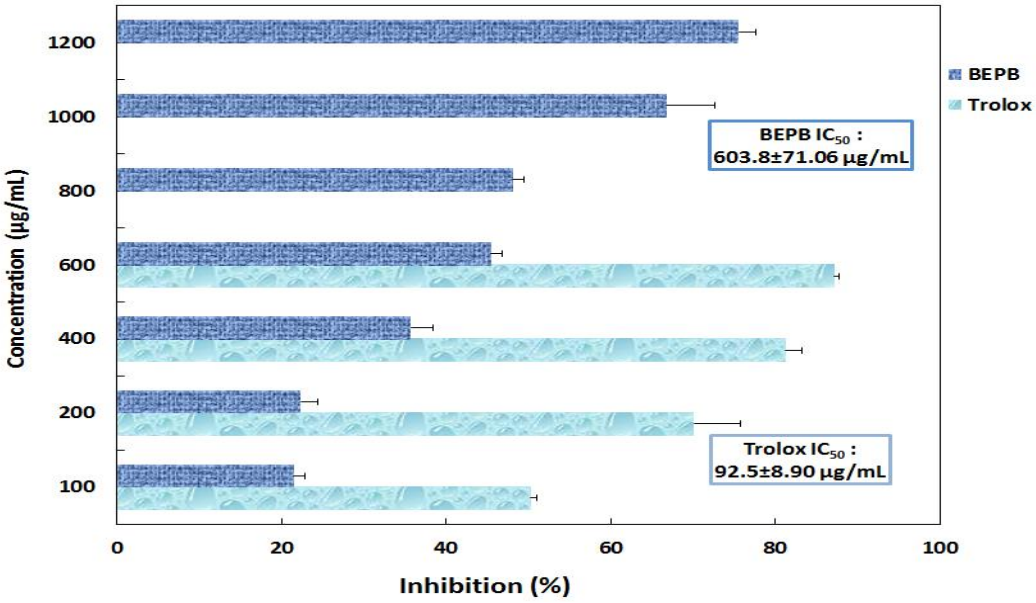


Figure 3: H_2O_2 scavenging ability of BEPB and trolox as standard. Each value represents a mean ± SD (n=3), P<0.05.

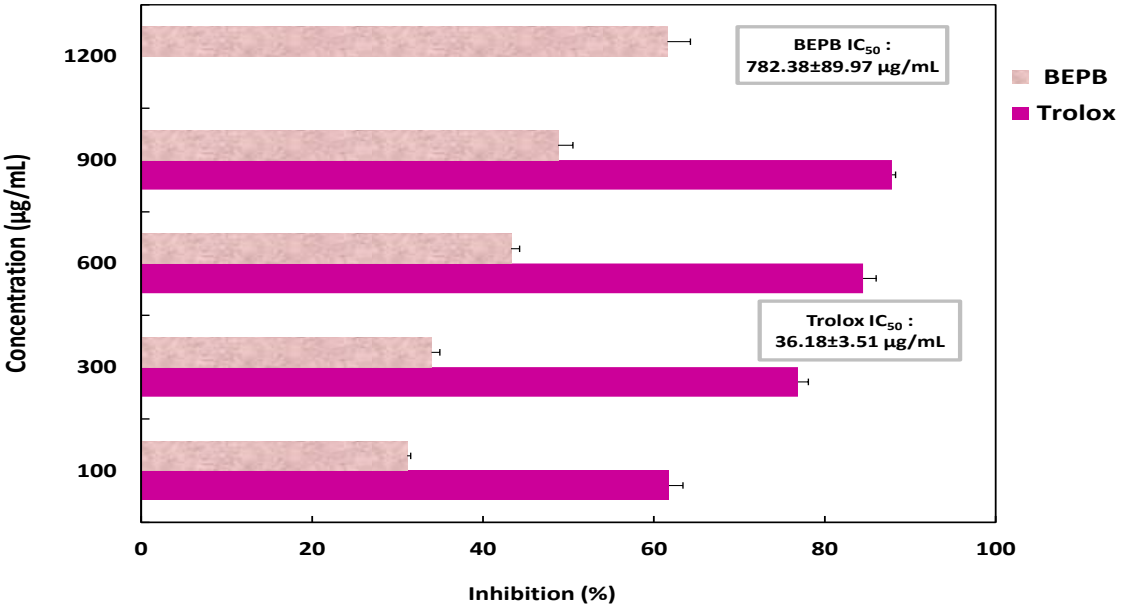


Figure 4: Antihemolytic property of BEPB and trolox as standard. Each value represents a mean ± SD (n=3), P<0.05.

only 300 µg/mL and 200 µg/mL for 83.36% and 98% DPPH• scavenging effect, respectively. The radical scavenging activity of BEPB was much lower (IC_{50} 876.16±7.96 µg/mL) than those of both standard references (trolox IC_{50}: 30.08±0.83 µg/mL; ascorbic acid IC_{50}: 6.41±0.61 µg/mL), suggesting that the antioxidant effect of BEPB extract was moderate and required high concentration to have a significant effect (Figure 2).

H_2O_2 scavenging activity of BEPB

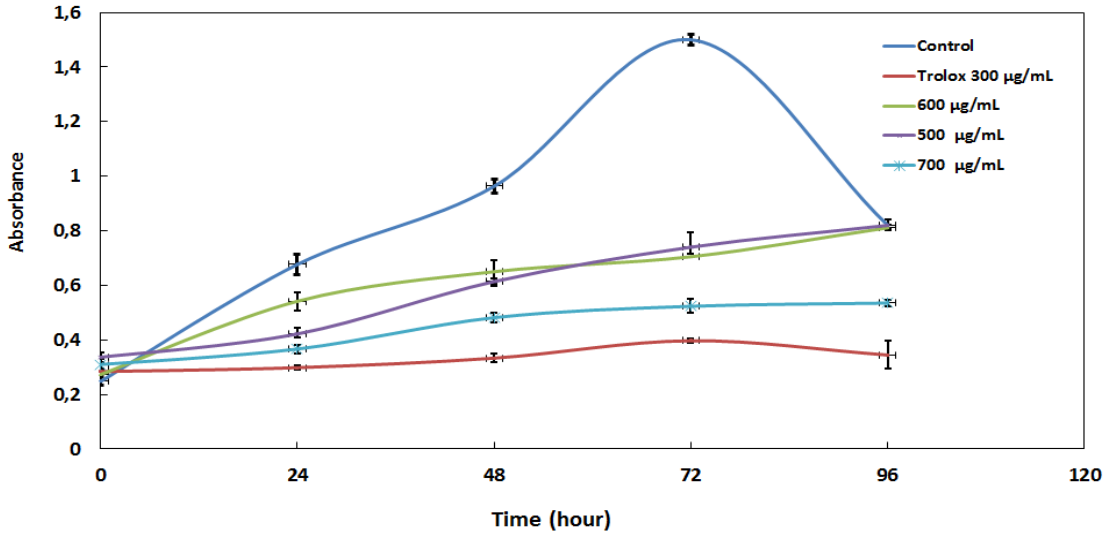
Despite that H_2O_2 was considered poorly reactive because of its weaker oxidizing capabilities, it can produce cytotoxicity by generating hydroxyl radical (OH•) that can initiate lipid peroxidation[10,11]. H_2O_2 scavenging activity of the BEPB was also concentration dependent, BEPB also possess significant ability to quench the hydrogen peroxide, the H_2O_2 scavenging activity was found to be maximum (66.81-75.60%) at the concentration ranging between 1000-1200 µg/mL with IC_{50}: 603.8±71.06 µg/mL. Trolox which was taken as a reference compound shows 81.21% H_2O_2 inhibition at a concentration of 400 µg/mL with IC_{50}: 92.5±8.90 µg/mL (Figure 3).

Antihemolytic property of BEPB

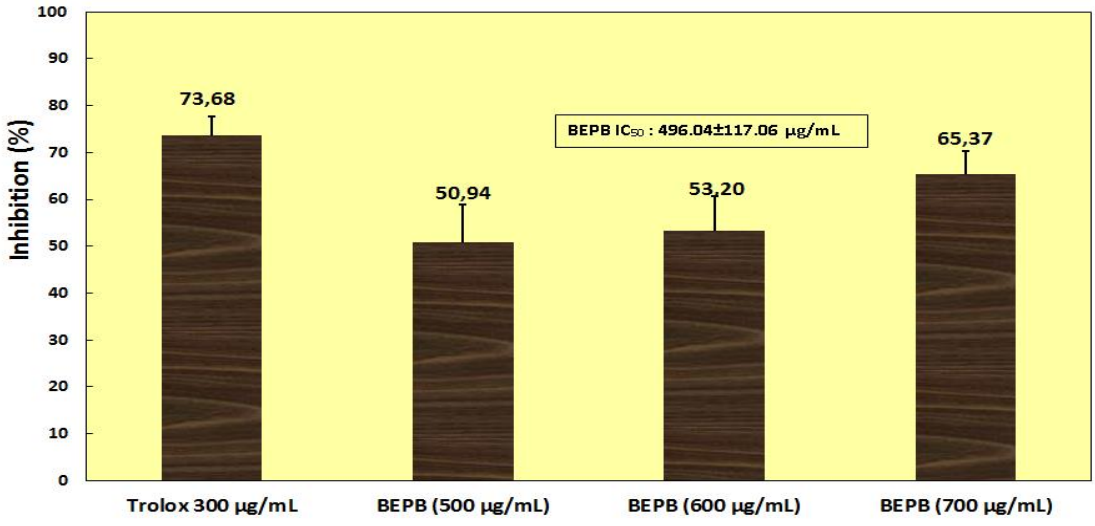
Hemolysis is the rupture of red blood cells membrane, causing the liberation of hemoglobin and other components into the extra cellular fluid[15]. The H_2O_2 erythrocyte hemolytic process may be explained by the penetrating of H_2O_2 in cellular membranes that triggers hydroxyl radical (OH•) formation in the presence of metal ions[5], the Hydroxyl radicals which eliminate hydrogen atoms from the membrane lipids leads to lipid peroxidation and hemoglobin leakage[51]. The results of the present study clearly indicate that BEPB inhibited rat erythrocytes hemolysis induced by H_2O_2 in a dose dependent way. The maximum percentage inhibition (61.58 %) showed by BEPB was at a concentration of 1000-1200 µg/mL, with IC_{50} value: 782.38±89.97 µg/mL (Figure 4), the standard reference, trolox exhibited 87.85% hemolysis inhibition at 700 µg/mL with IC_{50} value: 36.18 ± 3.51 µg/mL (Figure 4).

Inhibition of lipid peroxidation of BEPB

One of oxidative stress damages is lipid peroxidation that produces lipid peroxides[6]. Lipid peroxidation usually proceed as a chain reaction; followed in the propagation



(a)



(b)

Figure 5: Antilipid peroxidation effect of BEPB and trolox as standard (a), inhibition percentage of antiperoxidative effect (b). Each value represents a mean ± SD (n=3), P<0.05.

phase, by the formation of reactive species, and terminating with the formation of lipid hydroperoxide (ROOH)[52,53]. In our study, inhibition of lipid peroxidation activity of BEPB was estimated by the method of thiocyanate by using linoleic acid as a source of peroxide[50]. The generated peroxide reacted with the ferrous chloride (Fe^{2+}) to form the ferric ion (Fe^{3+}) monitor form of a thiocyanate complex. Linoleic acid, an unsaturated fatty acid, can be easily peroxided and produces various compounds such as aldehydes and epoxides. The present study monitored the amount of lipid peroxide formed every 24 hours, over a period of 4 days, by monitoring the colored complex of lipid peroxide which absorbs light at 500 nm. It was found that on the third day, the absorbance of the control solution reached a maximum indicating the maximum formation of lipid peroxides. Low absorbance value at 500 nm indicated high level of inhibition of lipid peroxidation and therefore high antioxidant activity. Figure 5, showed that the control OD has increased up to 1.50±0.03 at 72 hours, then it has decreased, at the same time the BEPB-OD at 700 µg/mL was recorded at 0.52±0.03, while the trolox as standard antiperoxidant exhibited only a 0.40±0.01 OD value at 300 µg/mL. The percentages of linoleic acid peroxidation inhibition for BEPB at all concentrations (500, 600, 700 µg/mL) were 50.94, 53.20, and 65.37%, respectively with IC_{50} value: 496.04±117.06 µg/mL as compared to trolox (73.68%) at 300 µg/mL (Figure 5 a and b).

Recapitulated results revealed that BEPB carry the antioxidative effect for free radical scavenging ($DPPH^{\bullet}$, H_2O_2), chain-breaking inhibition of lipid peroxidation and for hemolysis inhibition; these properties may be due to the combined activity of the above mentioned bioactive components with other components, most specifically, furanocoumarin (xanthotoxol), cacticin and mannitol, that have been previously reported to be responsible for the antioxidant and are proficient of donating hydrogen to a free radical ($DPPH^{\bullet}$ and H_2O_2) to convert them to non reactive species[54-57]. The structure activity relationship study showed that the antioxidant activity of the coumarin derivatives could be attributed to electron donating nature of the substituents like -OH, $-CH_3$ and -Cl on coumarin scaffold, reduce free radical and prevent the damage of cell[58-60]. Flavonoids have been extensively reported by many researchers to play an important role in the treatment of anemia, the binding of the flavonoids to the RBC membranes significantly inhibits lipid peroxidation, and at the same time enhances their integrity against lysis[15,61,62].

From our results it can be suggested that the antioxidant activity of BEPB might be due to synergy effect of the xanthotoxol, 8-(6',7'-dihydroxygeranyloxy)-psoralen, cacticin and mannitol present in the *n*-BuOH extract of *P. battandieri*, that have been separately shown to possess different biological effects[45,46,54-56].

CONCLUSION

Four compounds namely mannitol **1**, xanthotoxol **2**, 8-(6',7'-dihydroxygeranyloxy)-psoralen **3** and cacticin **4** have been isolated from the *n*-BuOH extract of *Pituranthos battandieri* Maire. These compounds are described for the first time for this species. On the basis of the results obtained in the present study, we conclude that the *n*-BuOH extract of *P. battandieri* possess antioxidant properties in all oxidation tested models ($DPPH^{\bullet}$, H_2O_2, H_2O_2-rat hemolysis, LPO-thiocyanate/linoleic acid) the antioxidative damage proved by *P. battandieri* appears as a consequence of synergistic interactions among a number of active constituents (furanocoumarins: xanthotoxol and 8-(6',7'-dihydroxygeranyloxy)-psoralen, cacticin and mannitol).

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