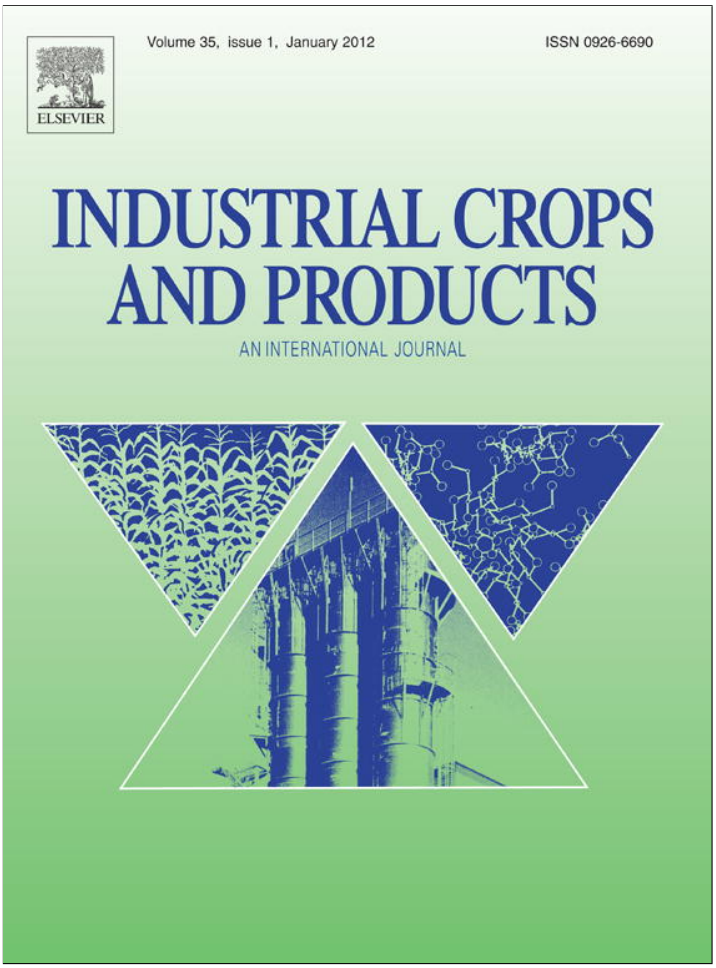

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Exploration of cholinesterase and tyrosinase inhibitory, antiprotozoal and antioxidant effects of *Buxus sempervirens* L. (boxwood)

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ABSTRACT

The petroleum ether, chloroform, ethyl acetate, and methanol extracts of the aerial parts of *Buxus sempervirens* growing naturally in Turkey were investigated against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) as well as tyrosinase (TYRO) enzymes for their inhibitory activity using ELISA microplate reader. Antiprotozoal activity of the extracts was tested against the parasites; *Plasmodium falciparum* (malaria) and *Trypanosoma brucei rhodesiense* (human African trypanosomiasis) at 0.81 and 4.85 μg/mL concentrations. As antioxidant activity contributes to antimalarial activity, the extracts were also tested for their 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, iron-chelating capacity, and ferric-reducing antioxidant power (FRAP). Total phenol and flavonoid contents in the extracts were determined spectrophotometrically. The alkaloid fraction of the chloroform extract afforded two major alkaloids named (+)-buxabenzamidienine (**1**) and (+)-buxamidine (**2**). Our results showed that, except for the petroleum ether extract, the extracts displayed high antimalarial and anti-BChE effects as well as quite high iron-chelation capacity. Therefore, we suggest that the active extracts in the antimalarial tests might be showing their effects through the mechanism of BChE inhibition and additionally iron-chelation ability.

1. Introduction

Buxus sempervirens L. (Buxaceae), known as "box wood, common box or European box" in English, is a wild edible plant species native to Europe, northwest Africa and South Asia. It is an evergreen shrub or small tree and also popular as ornamental plant in gardens. Besides its medicinal utilization of *B. sempervirens*, it has been used for its residential vegetation features in urban landscapes in Turkey (Acar et al., 2007) and because its strong wood, the plant is used in making hairbrush traditionally in Turkey as well as hedge, topiary, boxes, cabinet, and other woodcraft production. The plant has different traditional uses in folkloric medicine. For instance; the tea form of *B. sempervirens*, named as "şimşir" in Turkish, is drunk a glass *per* day prior to meals for antihelminthic, diaphoretic, and cholagogue purposes in Anatolia (Baytop, 1999). The bark can be harvested at any time of the year and is dried for its use to prepare decoctions. A tincture of the wood has been used as a bitter tonic and antiperiodic, it has also had a reputation for curing leprosy (Bown, 1995). The leaves of *Buxus hyrcana* have been reported to be used as powder or ointment against some skin conditions such as skin wounds, skin irritations, allergic rashes, and dermatitis in Russia and central Asia (Mamedov et al., 2005). The leaves, bark, and root of *B. sempervirens* have been recorded to be utilized as traditional medicine against malaria in the northern Portugal (Neves et al., 2009). The phytochemical content of the plant has been dominated by alkaloids (Atta-ur-Rahman et al., 1989, 2001; Loru et al., 2000) as well as some triterpenoids and sterols (Abramson et al., 1973; Desai et al., 1981), and anthocyanins (Lee, 2002).

On the other hand, malaria is one of the most important parasitic infections in humans due to its elevated morbidity and mortality with main resultant impact on economic output and income and approximately 40% of the world population is under risk of developing malaria (Salom-Roig et al., 2005). Various antimalarial drugs such as quinine, mepacrine, and paludrine have been reported to act on cholinesterases through inhibition of those enzymes (Blaschko et al., 1947; Wright and Sabine, 1948). Besides, insecticidal and anthelmintic activity is also strongly associated with cholinesterase inhibition and, therefore, many insecticidal and anthelmintic drugs show their effect through this mechanism (Hart and Lee, 1966; Knowles and Casida, 1966; Winteringham, 1966; Darlington et al., 1971). Taking traditional uses of the plant into account, the present study was undertaken to investigate cholinesterase inhibitory activity of the petroleum ether, chloroform, ethyl

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acetate, and methanol extracts of the aerial parts of *B. sempervirens* growing naturally in Turkey against acetylcholinesterase (AChE, true cholinesterase) and butyrylcholinesterase (BChE, pseudocholinesterase) enzymes by high throughput screening method using ELISA microplate reader. Considering its use against skin conditions, the same extracts were also screened against tyrosinase (TYRO), an enzyme important in pathogenesis of some skin diseases such as hyperpigmentation and melasma. Due to ethnopharmacological use of the plant against malaria, the extracts were tested against the malaria vector; *Plasmodium falciparum* as well as *Trypanosoma brucei rhodesiense*; the factor of human African trypanosomiasis. Antioxidant activity of the extracts was established in three *in vitro* test systems; 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, iron-chelating capacity, and ferric-reducing antioxidant power (FRAP) tests. Besides, total phenol and flavonoid contents of the extracts were determined spectrophotometrically by Folin–Ciocalteau's and aluminum chloride reagents, respectively. In addition, the alkaloid fraction obtained from the chloroform extract of the plant was subjected to chromatographic separation, which led to isolation of two major alkaloids.

2. Materials and methods

2.1. Plant material

The aerial parts of *B. sempervirens* L. were collected from the vicinity of Giresun province located at the northeastern part of Turkey in May, 2005. The plant was identified by Prof. Dr. Bilge Sener of Department of Pharmacognosy, Faculty of Pharmacy, Gazi University (Ankara, Turkey). A voucher specimen (AEF 23655) of the plant is preserved at the Herbarium of Faculty of Pharmacy of Ankara University (Ankara, Turkey).

2.2. Preparation of extracts and the alkaloid fraction

Following collection, the whole aerial parts without flowers were air-dried, cut into small pieces, and ground in a grinder. Then, the powdered material (88.64 g) was firstly extracted with petroleum ether (PE), followed by chloroform ($CHCl_3$), ethyl acetate (EtOAc), and methanol (MeOH), and each organic phase was concentrated *in vacuo* to obtain the crude extracts. Presence of the alkaloid-type of compounds was checked using Dragendorff's reagent *via* thin layer chromatography (TLC) and only the chloroform extract was observed to contain alkaloidal compounds due to orange-colored spots on TLC plate, which was developed in the solvent system consisting of chloroform:methanol (9:1). Then, the alkaloid fraction was prepared from the chloroform extract according to classical acid-base shifting method, adjusting pH of the extract to 11 and presence of the alkaloids in the fraction was confirmed using Dragendorff's reagent again in the same manner.

2.3. Cholinesterase inhibition tests

AChE and BChE inhibitory activity of the samples was measured by slightly modifying the spectrophotometric method developed by Ellman et al. (1961). *Torpedo californica* (electric eel) AChE (Type-VI-S, EC 3.1.1.7, Sigma) and horse serum BChE (EC 3.1.1.8, Sigma) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as substrates of the reaction. 5,5′-Dithio-bis(2-nitrobenzoic)acid (DTNB, Sigma, St. Louis, MO, USA) was used for the measurement of the cholinesterase activity. Briefly, in this method, 0.1 mM sodium phosphate buffer (pH 8.0), DTNB, test solution, and AChE/BChE solution were added by multichannel automatic pipette (Gilson pipetman, France) in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of the substrates; acetylthiocholine iodide/butyrylthiocholine chloride, respectively. Hydrolysis of the substrates was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm utilizing a 96-well microplate reader (VersaMax Molecular Devices, CA, USA). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software. Percentage of inhibition of AChE/BChE was determined by comparison of rates of reaction of samples relative to blank sample (ethanol in phosphate buffer pH = 8) using the formula $(E - S)/E \times 100$, where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Galanthamine (Sigma, St. Louis, MO, USA), the anticholinesterase alkaloid-type of drug isolated from the bulbs of snowdrop (*Galanthus* sp.), was used as reference.

2.4. Tyrosinase inhibitory activity test

Inhibition of mushroom tyrosinase (EC 1.14.1.8.1, Sigma) by the samples was determined using the modified dopachrome method with L-DOPA as substrate (Masuda et al., 2005). Assays were conducted in a 96-well microplate and an ELISA microplate reader (VersaMax Molecular Devices, USA) was used to measure absorbance at 475 nm. The extracts dissolved in DMSO with phosphate buffer (pH 6.8), tyrosinase, and L-DOPA were put in each well. Each sample was accompanied by a blank that had all the components except for L-DOPA. Results were compared with a control consisting of 50% DMSO in place of sample. The percentage tyrosinase inhibition was calculated as follows:

$$\%\ \text{Inhibition} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software.

2.5. Antitrypanosomal activity test

Minimum essential medium supplemented according to Baltz et al. (1985) with 2-mercaptoethanol and 15% heat-activated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were prepared within a range between 90 and 0.123 μg/mL. Afterward, 10^4 bloodstream forms of *T. brucei rhodesiense* STIB 900 were added into each well and the plate incubated at 37 °C under a 5% CO_2 atmosphere for 72 hours. Alamar Blue dye was then added to each well again and incubation was applied for another 2–4 h. Following this duration, the plate was read in a ELISA microplate reader (SpectraMax Gemini XS microplate fluorometer, Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm (Räz et al., 1997). Fluorescence development was expressed as percentage of the control.

2.6. Antimalarial activity test

Antimalarial activity was tested against the K1 strain of *P. falciparum* (resistant to chloroquine and pyrimethamine) by a modified [^{3}H]-hypoxanthine incorporation method (Matile and Pink, 1990). To sum up, infected human red blood cells in RPMI 1640 medium with 5% Albumax were exposed to serial drug dilutions in microplates. Following 48 hours of incubation at 37 °C in a reduced oxygen atmosphere, 0.5 μCi ^{3}H-hypoxanthine was put into each well. Cultures were incubated for another 24 h before they were harvested. The radioactivity was counted using a Betaplate™ liquid scintillation counter (Wallac, Zurich, Switzerland). The results were calculated as counts *per* minute (CPM) *per* well at each concentration and expressed as percentage of the untreated controls.

2.7. Antioxidant activity

2.7.1. DPPH radical scavenging activity

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined by Blois method (1958). The samples dissolved in methanol (75%) were mixed with DPPH solution (1.5×10^{-4} M). Remaining DPPH amount was measured at 520 nm using a Unico 4802 UV–visible double beam spectrophotometer (USA). Gallic acid and butylated hydroxyanisol (BHA) were employed as the references. Inhibition of DPPH in percent ($I\%$) was calculated as given below:

$$I\% = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts/reference. Analyses were run in triplicates and the results were expressed as average values with S.E.M. (standard error mean).

2.7.2. Iron-chelating effect

The iron-chelating effect of the samples was estimated by the method of Chua et al. (2008). Briefly, the samples were incubated with 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 5 mM ferrozine into the mixture and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. The ratio of inhibition of ferrozine–Fe^{2+} complex formation was calculated as follows:

$$I\% = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

where A_{blank} is the absorbance of the control reaction (containing only $FeCl_2$ and ferrozine), and A_{sample} is the absorbance of the extracts/reference. Analyses were run in triplicates and the results were expressed as average values with S.E.M.

2.7.3. Ferric-reducing antioxidant power assay (FRAP)

The ferric-reducing power of the samples was tested using the assay of Oyaizu (1986). Different concentrations of the samples (1 mL) were added into 2.5 mL of phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide. Later, the mixture was incubated at 50 °C for 20 min and then 10% trichloroacetic acid was added. After the mixture was shaken vigorously, this solution was mixed with distilled water and $FeCl_3$ (0.1%, w/v). After 30 min incubation, absorbance was read at 700 nm using a Unico 4802 UV–visible double beam spectrophotometer (USA). Analyses were achieved in triplicate. Increased absorbance of the reaction meant increased reducing power.

2.7.4. Determination of total phenol and flavonoid contents

Phenolic content in the extracts was determined according to Folin–Ciocalteau's method (Singleton and Rossi, 1965). In brief, the extracts were mixed with 750 μL of Folin–Ciocalteau's reagent (Sigma) and 600 μL of sodium carbonate in test tubes. The tubes were then vortexed and incubated at 40 °C for 30 min. Afterward, absorption was measured at 760 nm at a Unico 4802 UV–visible double beam spectrophotometer (USA). Total flavonoid content of the extracts was calculated by aluminum chloride colorimetric method (Woisky and Salatino, 1998). To sum up, a number of dilutions of quercetin were obtained to prepare a calibration curve. Then, the same dilutions from the extracts were also prepared and separately mixed with 95% ethanol, 10% aluminum chloride, 1 M sodium acetate, and distilled water. Following incubation for 30 min at room temperature, absorbance of the reaction mixture was measured at wavelength of 415 nm with a Unico 4802 UV–visible double beam spectrophotometer (USA). The total phenol and flavonoid contents of the extracts were expressed as gallic acid and quercetin equivalents (mg/g extract), respectively.

2.7.5. Isolation and identification of the alkaloids

The alkaloid fraction obtained from the chloroform extract of the aerial parts of *B. sempervirens* was applied to Si 60 column chromatography (CC) eluting with dichloromethane:methanol and 44 fractions were collected. Following TLC monitoring, the similar fractions were combined again and 7 main subfractions (Sfr.s I to VII) were obtained. Among these fractions, Sfr.II was subjected to silica gel 60 (Si 60) CC using the similar elution system and further 9 subfractions were collected, which were later combined into 5 more subfractions (Sfr.II-1 to II-5). Out of these subfractions, Sfr.II-2 was further purified by washing with methanol and the precipitate was collected in a pure form (Compound **1**, 13.8 mg).

Another main fraction (Sfr.IV) was loaded to medium pressure liquid chromatography (MPLC) in a Master Flex L/S Digital Economy Drive MPLC pump equipped with Spectra/Chrom 1.5 c × 25 c LC Column using Foxy 200 Fraction Collector eluting with the mixtures of MeOH:H_2O. The column packing material was LiChropep RP-18 (25-40 UM) (Merck-109303). This separation led us to collect 130 subfractions in total. After checking the TLC profiles of these subfractions, they were classified into 5 new subfractions. Among them, the last subfraction (101–130) afforded compound **2** (14.5 mg) in a pure form through preparative TLC using the solvent system consisting of hexane:chloroform (75:25). In fact, presence of these two alkaloids was found in all of the subfractions.

Spectral analysis of the pure compounds **1** and **2** was achieved using Varian Mercury 400, 400 MHz High Performance Digital FT-NMR Spectrometer for ^1H and ^{13}C NMR spectra in $CDCl_3$, while mass spectrum was taken in LC–MS Waters 2695 Alliance Micromass ZQ apparatus. Comparison of our one- and two-dimensional NMR data reflected an appropriate match to the previously reported data and the compounds **1** and **2** were elucidated as (+)-buxabenzamidienin and (+)-buxamidine (Kurakina et al., 1972; Atta-ur-Rahman et al., 1989) (Fig. 1).

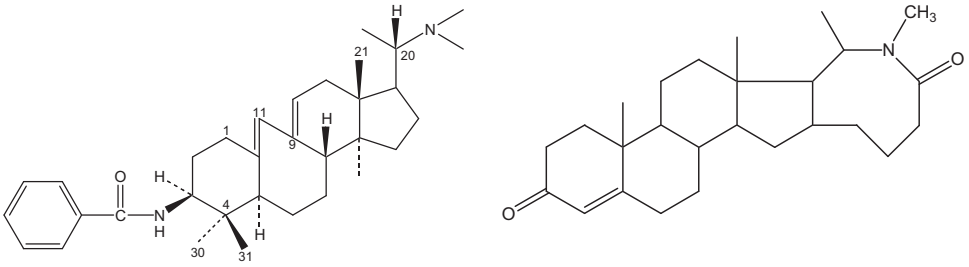
3. Results

As tabulated in Table 1, the MeOH extract displayed the highest inhibition (58.6 ± 1.58%) against AChE at 100 μg/mL, while the best inhibition (91.4 ± 1.31%) was caused by the EtOAc extract at the same concentration, followed by the MeOH extract (86.1 ± 0.86%). On the other hand, all of the extracts exerted insignificant inhibition against TYRO below 50% at the tested concentrations (Table 1).

Antiprotozoal activity results of the extracts are listed in Table 2. Accordingly, the extracts showed low activity at 0.81 μg/mL against both *P. falciparum* and *T. brucei rhodesiense*, whereas they had a high antiprotozoal effect on *P. falciparum* at 4.85 μg/mL except for the PE extract.

The calibration equations were calculated as $y = 1.065 + 0.0321$ ($r^2 = 0.9778$) for total phenol and $y = 0.006x - 0.032$ ($r^2 = 0.9946$) for total flavonoid contents. The MeOH extract was found to be the richest in total phenol content as gallic acid equivalent, whereas it contained the lowest total flavonoid content as seen in Table 3. On the other hand, occurrence of low radical scavenging activity against DPPH was observed in the extracts (Table 3).

The extracts possessed a significant level of iron-chelating capacity (Fig. 2), which was higher than the reference butylated hydroxyanisol (BHA) at the tested concentrations. On the other hand, all of the extracts exerted a low FRAP as compared to the reference; chlorogenic acid (Fig. 3).



1 ($C_{33}H_{48}ON_2$) **2** ($C_{26}H_{39}NO_2$)

Fig. 1. Compounds **1** and **2** isolated from the alkaloid fraction of *B. sempervirens*.

4. Discussion

The data obtained from this study confirms the antimalarial use of *B. sempervirens* in folkloric medicine as its extracts displayed a high profile in antimalarial assays. It is obvious that the extracts that showed highest antimalarial activity in our tests displayed the highest inhibition against BChE (Tables 1 and 2). Since many antimalarial drugs act through cholinesterase inhibition as afore-mentioned, it might be speculated that mode of action of the extracts with antimalarial activity could be partly connected to their BChE inhibitory potential. This can be also claimed due to the findings that antimalarial drugs were reported to inhibit cholinesterase enzyme family, being pseudocholinesterase (BChE) the main-inhibited enzyme rather than AChE (Blaschko et al., 1947; Wright and Sabine, 1948), which is in accordance with our results.

Table 1
Inhibition % of *Buxus sempervirens* extracts against AChE, BChE, and TYRO.

Extracts	Inhibition % ± S.E.M.[a]					
	AChE		BChE		TYRO	
	25 μg/mL	100 μg/mL	25 μg/mL	100 μg/mL	25 μg/mL	100 μg/mL
Petroleum ether	–[b]	–	–	23.7 ± 1.89	3.17 ± 0.62	6.87 ± 0.24
Chloroform	16.2 ± 2.79	27.5 ± 2.27	56.2 ± 3.44	58.9 ± 2.05	2.10 ± 0.02	6.61 ± 0.76
Ethyl acetate	14.7 ± 2.11	29.7 ± 0.54	48.3 ± 2.32	91.4 ± 1.31	1.76 ± 0.11	6.96 ± 1.36
Methanol	29.4 ± 2.76	58.6 ± 1.58	63.9 ± 1.07	86.1 ± 0.86	14.84 ± 1.15	16.70 ± 0.35
Galanthamine (reference for AChE and BChE)	99.9 ± 0.31	100.0 ± 0.64	80.3 ± 1.14	98.60 ± 0.59		
α-Kojic acid (reference for TYRO)					42.34 ± 1.03	78.89 ± 0.09

[a] Standard error mean ($n = 3$).
[b] No inhibition.

Table 2
Antiprotozoal activity (%) of *Buxus sempervirens* extracts.

Extracts	*Plasmodium falciparum* (Malaria)		*Trypanosoma brucei rhodesiense* (Human African Trypanosomiasis)	
	0.81 μg/mL	4.85 μg/mL	0.81 μg/mL	4.85 μg/mL
Petroleum ether	1.8	31.7	17.1	9.9
Chloroform	17.3	87.3	–[a]	4.0
Ethyl acetate	7.0	79.2	4.5	3.0
Methanol	19.1	69.1	4.6	14.5
Melarsoprol[b]			–[c]	99.5
Artemisinin[d]	–	99.7		

[a] No activity.
[b] Reference against *Trypanosoma brucei rhodesience*.
[c] Not determined.
[d] Reference against *Plasmodium falciparum*.

Table 3
Total phenol and flavonoid contents ± S.E.M. and DPPH radical scavenging activity (inhibition % ± S.E.M.) of *Buxus sempervirens* extracts.

Extracts	Total phenol[a] contents ± S.E.M.[b]	Total flavonoid[c] contents ± S.E.M.	DPPH radical scavenging activity (inhibition % ± S.E.M.)			
			250	500	1000	2000
PE	12.0 ± 4.24	84.84 ± 4.51	3.44 ± 0.09	4.98 ± 0.78	7.56 ± 0.01	12.96 ± 1.91
$CHCl_3$	52.58 ± 5.94	75.84 ± 6.84	3.44 ± 0.09	4.66 ± 0.09	7.36 ± 0.47	13.45 ± 1.84
EtOAc	40.61 ± 3.65	82.50 ± 1.65	4.32 ± 0.38	6.41 ± 0.10	11.07 ± 0.95	14.38 ± 0.86
MeOH	63.85 ± 0.66	37.55 ± 1.59	4.32 ± 0.95	6.48 ± 1.91	11.41 ± 1.05	23.01 ± 2.57
Gallic acid			–[d]	91.61 ± 0.06	92.57 ± 0.10	98.76 ± 0.23

[a] Data expressed in mg equivalent of gallic acid (GAE) to 1 g of extract.
[b] Standard error mean.
[c] Data expressed in mg equivalent of quercetin to 1 g of extract.
[d] Not determined.

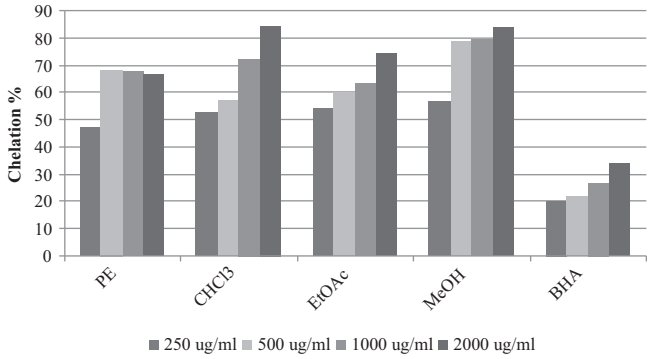


Fig. 2. Iron-chelating capacity (inhibition %) of *Buxus sempervirens* extracts.

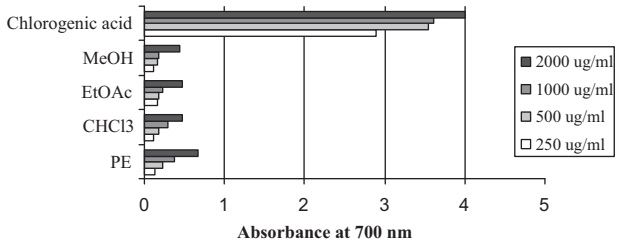


Fig. 3. FRAP graph of *Buxus sempervirens* extracts.

Another interesting outcome of our study is high iron-chelating capacity of the active extracts. Because having iron-chelation capacity is a great advantage for any antimalarial drug as the combined therapy of iron-chelators and classical antimalarials constitutes a new approach in malaria treatment (Van Zyl et al., 1992). For instance; some antimalarials such as dexrazoxane (Loyevski et al., 1999), dicatecholate (Pradines et al., 2002), and siderophore (Pradines et al., 2006) were reported to have iron-chelating effect which contributed an additive effect on antimalarial activity of these compounds. In fact, antioxidant activity leads occurrence of higher antimalarial activity in some cases such as artesunate (Arreesrisom et al., 2007).

In addition, our experimental findings indicated that there seems to be no direct correlation between the bioactivities performed in the present study and total phenol and flavonoid contents of the extracts. In fact, *B. sempervirens* has been well-known to be quite prosperous in alkaloid-type of compounds. This may lead another assumption that not phenolic, but alkaloidal compounds found in *B. sempervirens* could be responsible for the high anticholinesterase and antimalarial activities. Because, the *Buxus* genus including *B. sempervirens* (Kvaltinova et al., 1991), *Buxus papillosa* (Atta-ur-Rahman et al., 2001), and *B. hyrcana* (Choudhary et al., 2003) was reported to contain cholinesterase-inhibiting alkaloids. Since we isolated two major alkaloids (**1** and **2**) from the alkaloid-rich fraction of the chloroform extract of the plant having the highest antimalarial activity, the alkaloids might be responsible for the mentioned activity in this extract.

5. Conclusion

As a conclusion, the screening results suggest that the ethnopharmacological use of *B. sempervirens* against malaria can be confirmed on a scientific base and the plant deserve further studies to be evaluated as promising antimalarial agent. To the best of our knowledge, this is the first study on antiprotozoal activity and iron-chelation capacity of *B. sempervirens*.

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