SHORT REPORT

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Rec. Nat. Prod. 9:1 (2015) 159-163

records of natural products

Chemical Constituents and Antioxidant Activity of *Teucrium barbeyanum* Aschers

Mohamed Ali A. Alwahsh, Melati Khairuddean* **and Wong Keng Chong**

School of Chemical Sciences, Universiti Sains Malaysia, 11800, Penang, Malaysia

(Received September 30, 2013; Revised March 10, 2014; Accepted July 8, 2014)

Abstract: The different extracts of the aerial parts of *Teucrium barbeyanum* Aschers. were investigated for the chemical constituents and antioxidant activities. The chemical investigation of the plant led to the isolation of eleven known compounds through column chromatography in which nine were flavonoids and the other two were simple phenolic compounds. The compounds were characterized using NMR techniques (^{1}H, ^{13}C, DEPT-135 and 90, COSY, HMQC, HMBC and NOESY), UV spectroscopy and EI/ESI spectrometry. The isolated compounds were identified as 5-hydroxy-3,6,7,4′-tetramethoxyflavone (**1**), salvigenin (**2**), 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**3**), chrysosplenetin (**4**), cirsilineol (**5**), cirsimaritin (**6**), cirsiliol (**7**), apigenin (**8**) and luteolin (**9**), in addition to methyl caffeate (**10**) and 4-hydroxybenzoic acid (**11**). The antioxidant activity of the extracts was evaluated using 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and ferric reducing antioxidant power (FRAP) methods. Ethyl acetate and butanol extracts showed comparable antioxidant activity to known antioxidants; trolox and ascorbic acid and the highest total phenolic and flavonoid contents. The active components were extracted efficiently in 70% aqueous methanol after defatting procedure. This is the first time the aforementioned compounds are isolated from this plant, and there has been no previous report on the biological studies on this species.

Keywords: *Teucrium barbeyanum* Aschers.; chemical constituents; flavonoids; antioxidant activity.

1. Plant Source

The aerial parts of *Teucrium barbeyanum* Aschers. were collected from mountainous slopes of "Ras El-Hilal" in "Gebel Akhdar", Libya during the flowering season in May 2012. A voucher specimen (USM 11473) has been deposited in the herbarium of Universiti Sains Malaysia.

2. Previous Studies

Teucrium barbeyanum is an endemic perennial herb which grows in Al-Jabal Al-Akhdar (the Green Mountain) in Cyrenaica region of Libya [1]. The previous phytochemical study on the plant was carried out by Bruno et al. (1985). The study revealed the presence of three neo-clerodane diterpenoids [2]. However, there are no chemical reports regarding other phytochemical as well as biological

* Corresponding author: E-Mail: melati@usm.my; Phone:+604-6533560 *Fax:*+604-6574854

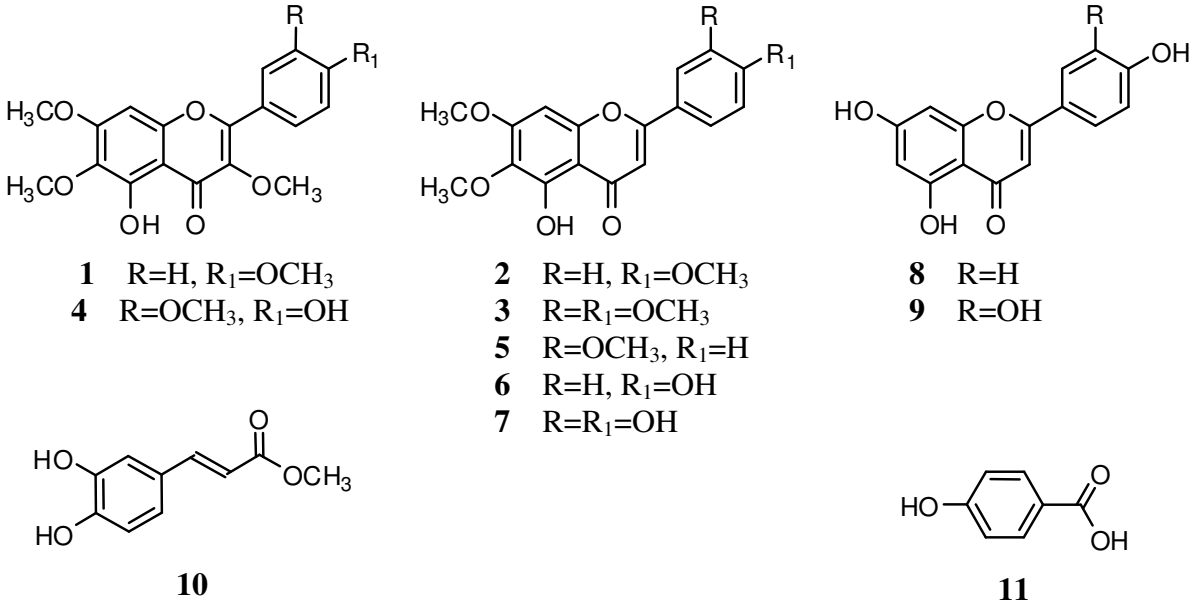
The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 09/23/2014 EISSN: 1307-6167

studies of this species. The species of this genus have been extensively used as diuretic, diaphoretic, antiseptic, antipyretic, antispasmodic and hypoglycemic agents in traditional medicine through the history. The pharmacological studies revealed that *Teucrium* has a variety of biological activities such as anti-inflammatory, antioxidant, antimicrobial, antihelminthic and antifeedant properties [3].

3. Present Study

1.2 kg of air dried and powdered aerial parts of the plant were defatted using petroleum ether (40-60 °C) with Soxhlet apparatus to afford the petroleum ether extract (Pet. ether ext.). The defatted plant material was further extracted with dichloromethane to obtain dichloromethane extract (DCM ext.). Afterwards, the plant material were exhaustively macerated with 70% aqueous methanol to give methanol extract after defatting (Meaf ext.), which was successively partitioned with chloroform, ethyl acetate and *n*-butanol to give chloroform extract ($CHCl_3$ ext.), ethyl acetate extract (EtOAc ext.) and butanol extract (BuOH ext.), respectively. The aqueous residual was considered as residue extract (Res. ext.). The extraction was also carried out with other strategies in which two separate quantities (10 g each) of the dried plant were macerated for 24 h. The first portion upon direct extraction with 70% aqueous methanol gave the methanol extract (Medi ext.) while the second portion was extracted with distilled water to give the water extract (water ext.). The extracts were concentrated to dryness using rotary evaporator at 40 °C under reduced pressure and were kept in freezer to be used.

The extracts of Pet. ether, DCM, $CHCl_3$ and EtOAc were subjected to subsequent column chromatography with solvent systems in a polarity gradient manner on silica gel as stationary phase to isolate the major constituents of each extract. Sephadex LH-20 was used for isolation and final purification of the compounds. The purified compounds were characterized using 1D and 2D NMR techniques, UV spectroscopy, and mass spectrometry techniques (EI/ESI) as 5-hydroxy-3,6,7,4′-tetramethoxyflavone (**1**), salvigenin (**2**), 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**3**), chrysosplenetin (**4**), cirsilineol (**5**), cirsimaritin (**6**), cirsiliol (**7**), apigenin (**8**), luteolin (**9**), methyl caffeate (**10**) and 4-hydroxybenzaoic acid (**11**) (**Figure 1** and supporting information **S1**-**S30**). To the best of the authors knowledge, this is the first report on the isolation of **1** and **4** from *Teucrium* genus.



1 R=H, R_1=OCH_3
4 R=OCH_3, R_1=OH

2 R=H, R_1=OCH_3
3 R=R_1=OCH_3
5 R=OCH_3, R_1=H
6 R=H, R_1=OH
7 R=R_1=OH

8 R=H
9 R=OH

10

11

Figure 1. Chemical structures of isolated compounds (**1-11**)

Antioxidant Activities Assays: The antioxidant activities of *T. barbeyanum* extracts were evaluated by DPPH, ABTS (called trolox equivalent antioxidant capacity, TEAC) and FRAP assays according to the methods described by Thaipong et al. (2006) [4]. The ability of the samples to scavenge the free radicals was assayed using $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ radicals while Fe^{3+}-TPTZ was used to evaluate the

reducing power of the samples through reducing Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ [5]. The three methods use 150 µl of samples and 2850 µl of the working solutions of DPPH, ABTS and Fe^{3+}-TPTZ, respectively. %Inhibition of DPPH and ABTS radicals was calculated according to equation (1).

$$\%inhibition = \frac{A(control) - A(sample)}{A(control)} \text{ X } 100 \quad \text{.....(1)}$$

Where *A*(*control*) is the absorbance of control and *A*(*sample*) is the absorbance of sample. Trolox, ascorbic acid and gallic acid were used as standards in DPPH assay while gallic acid and luteolin were used as standards in ABTS assay. The results were expressed as IC_{50} (µg/ml) and further reported in TEAC for ABTS assay. TEAC results were calculated by dividing slope (inhibition/concentration) of the samples by the slope (inhibition/concentration) of trolox [6]. In FRAP assay, results were expressed in micromol trolox equivalent per gram (µM TE/g) of the extract.

Total Phenolic Content (TPC): TPC of the samples were evaluated using Folin-Ciocalteu reagent according to the method modified by Thaipong et al. (2006) [4], in which 150 µl of samples were diluted with 2400 µl of deionized water. Thereafter, 150 µl of Folin-Ciocalteu reagent (0.25 N) was added, mixed for 3 minutes and the reaction mixture was then neutralized with 300 µl of Na_2CO_3 (1 N). The final mixture was incubated for 2 h in the dark and UV absorbances were taken at 725 nm. Gallic acid was used as standard and results were reported in micromol gallic acid equivalent per gram (µM GAE/g) of the extract.

Total Flavonoid Content (TFC): TFC were measured by $AlCl_3$ colorimetric method described by Zhishen et al. (1999) [7], in which 0.5 ml of the samples were diluted with 4.5 ml dist. water, followed with 0.3 ml of $NaNO_2$ (5% w/v). After 5 minutes, 0.6 ml of $AlCl_3$ (10% w/v) was added and the mixture was incubated for another 6 minutes. Finally, 2.0 ml of NaOH (1 M) were added and the final volume was brought up to 10 ml with dist. water. UV measurements were then taken at 510 nm. The standard curve of quercetin was used to quantify the flavonoid contents of the samples. The results were reported as micromole quercetin equivalent per gram (µM QE/g) of the extract.

Statistical Analyses: One way ANOVA was performed using SPSS software. Duncan's test was used to analyze significant difference of means of IC_{50}, TEAC, TE, QE and GAE readings at $p<0.05$, while Pearson Correlation Coefficient (PCC) was used to measure the coherence between the results of TPC and TFC, and the three antioxidant assays. IC_{50} values were calculated using Graph Pad Prism 6.0 software. All measurements were carried out in triplicate and the mean values ± standard deviations (SD) were reported.

The three assays demonstrated the potential antioxidant ability of EtOAc, BuOH, Meaf and to some extent $CHCl_3$, and Medi extracts. In **Table 1**, DPPH assay through IC_{50} values showed comparable antioxidant potential of these extracts (EtOAc ext. 5.39 µg/ml, BuOH ext. 5.44 µg/ml, Meaf ext. 6.73 µg/ml, $CHCl_3$ ext. 7.69 µg/ml and Medi ext 7.89 µg/ml) to the known antioxidants; trolox (6.25 µg/ml) and ascorbic acid (4.57 µg/ml) with no significant difference ($p<0.05$). Results of ABTS assay given in **Table 1** indicated that EtOAc and BuOH extracts displayed the highest scavenging capacity with TEAC (0.570 and 0.562), which is consistent with their low IC_{50} values (5.41 and 5.61 µg/ml), respectively. **Figures S31** and **S32** depicted the scavenging behavior of the series of extracts and standards in different concentrations over DPPH and ABTS radicals. The antioxidant activities of the extracts in DPPH and ABTS assays were found to be in the following descending order: EtOAc ≈ BuOH >Meaf > $CHCl_3$ > Res. > DCM which was highly correlated to the TFC results (**Table 2**). High consistency between DPPH and ABTS results reflected in high PCC (r = 0.982), is similar to other researcher's finding [8]. FRAP confirmed the flavonoids domination by showing highest correlation (r = 0.986) with TFC. The maximum reducing power (4350.9 and 4269.1 µmol TE/g) were observed for BuOH and EtOAc extracts, respectively. DCM ext. showed the lowest antioxidant in all assays and also the least of phenolic and flavonoid content.

Table 1. Antioxidant potential of *T. barbeyanum* extracts with DPPH, ABTS and FRAP assays.

Extract	DPPH assay	ABTS assay		FRAP assay
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	TEAC (µg/ml)	µmol TE/g
Water ext.	$12.01^{d} \pm 0.14$	$15.10^{h} \pm 0.03$	$0.207^{b} \pm 0.001$	$1677.1^{c} \pm 6.5$
Medi ext.	$7.89^{c} \pm 0.06$	$9.78^{g} \pm 0.11$	$0.328^{c} \pm 0.002$	$2282.3^{e} \pm 5.2$
Meaf ext.	$6.73^{b,c} \pm 0.09$	$8.41^{f} \pm 0.08$	$0.368^{d} \pm 0.005$	$2561.6^{f} \pm 11.4$
DCM ext.	$77.96^{f} \pm 1.68$	$70.14^{j} \pm 1.24$	$0.034^{a} \pm 0.000$	$289.1^{a} \pm 35.5$
$CHCl_3$ ext.	$7.69^{c} \pm 0.24$	$7.28^{e} \pm 0.36$	$0.417^{e} \pm 0.022$	$1940.3^{d} \pm 8.4$
EtOAc ext.	$5.39^{b,c} \pm 0.08$	$5.41^{d} \pm 0.02$	$0.570^{f} \pm 0.004$	$4269.1^{g} \pm 6.3$
BuOH ext.	$5.44^{b,c} \pm 0.13$	$5.61^{d} \pm 0.09$	$0.562^{f} \pm 0.006$	$4350.9^{h} \pm 4.9$
Res. ext.	$24.50^{e} \pm 0.43$	$19.89^{i} \pm 0.28$	$0.177^{b} \pm 0.004$	$918.0^{b} \pm 14.0$
Gallic acid	$1.27^{a} \pm 0.02$	$0.71^{a} \pm 0.01$	$4.225^{h} \pm 0.064$	
Trolox	$6.25^{b,c} \pm 0.02$	$3.09^{c} \pm 0.02$		
Ascorbic acid	$4.57^{b} \pm 0.08$			
Luteolin		$2.39^{b} \pm 0.04$	$1.309^{g} \pm 0.016$	

Results are in mean ± SD for triplicate. Different upper case letters means significantly different at level $p<0.05$.

Table 2. Total phenolic and total flavonoid contents of extracts of *T. barbeyanum.*

Extract	Total phenolic content	Total flavonoids content
	µmol GAE/g	µmol QE/g
Water ext.	$1165.0^{c} \pm 30.9$	$1410.0^{d} \pm 45.8$
Medi ext.	$1330.4^{d} \pm 62.5$	$1421.7^{d} \pm 29.3$
Meaf ext.	$1897.5^{e} \pm 40.8$	$1576.7^{e} \pm 31.8$
DCM ext.	$445.0^{a} \pm 13.0$	$525.0^{a} \pm 21.8$
CHCl3 ext.	$2175.8^{f} \pm 57.6$	$1311.7^{c} \pm 42.5$
EtOAc ext.	$2704.2^{g} \pm 26.7$	$2773.3^{f} \pm 55.3$
BuOH ext.	$1957.9^{e} \pm 47.7$	$2966.7^{g} \pm 41.6$
Res. ext.	$1021.3^{b} \pm 29.4$	$785.0^{b} \pm 31.2$

Results are in mean ± SD for triplicate. Different upper case letters means significantly different at level $p< 0.05$

In **Table 2**, EtOAc ext. showed the highest TPC value of 2704.2 µM GAE/g, followed by the $CHCl_3$ ext. with 2175.8 µM GAE/g while for TFC, the BuOH ext. showed the highest TFC value of 2966.7 µM QE/g, followed by the EtOAc ext. with 2773.3 µM QE/g. The impact of TPC and TFC on the antioxidant activity of the extracts were also supported by high Pearson's correlations coefficient of TPC and TFC with TEAC (r = 0.935 and r = 0.915, respectively) (**Table S33**). The correlation coefficient of TPC with TFC (r = 0.785) indicated that around 61.6% of TPC are flavonoid components. From this study, it is evidenced that the active antioxidant materials could be efficiently extracted from the plant after removal of the non-polar components. This could be seen from the high

TPC (1897.5 µM GAE/g) and TFC (1576.7 µM QE/g) and the potential antioxidant activity of Meaf extract. ABTS assay displayed TEAC of 0.368 µg/ml while the extract has a reducing power of 2561.6 µmol TE/g in FRAP assay. The results also revealed a relatively weak scavenging activity of water ext. (IC_{50}s 12.01 µg/ml and 15.10 in DPPH and ABTS respectively) contrast to Medi and Meaf extracts, which were extracted with different strategies.

Acknowledgments

The authors would like to thank the Ministry of higher education, Libya for the scholarship, the Research University Grant (RU1001/PKIMIA/811129), Zahri Othman for NMR measurments, Mr. Abdusslam Elmogasapi and Mr. Baharuddin Sulaiman for collection and identification of the plant.

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