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EVALUATION OF CYTOTOXICITY AND RADICAL SCAVENGING ACTIVITIES OF FLAVONES ISOLATED FROM THE TWIGS OF *DODONAEA ANGUSTIFOLIA*

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ABSTRACT. *Dodonaea angustifolia* (commonly known as Kitikita in Amharic) is a medicinal plant widely used in traditional medicine for its diverse therapeutic properties. The twigs of the plant were extracted with 1:1 (methanol:dichloromethane mixture) and subjected to chromatographic techniques and yielded 8 pure compounds included: 5,7-dihydro-3,4′,6-trimethoxyflavone **1**, 5,6,7-trihydroxy-3,4'-dimethoxyflavone **2**, 5,7,4'-trihydroxy-3,6-dimethoxyflavone **3**, 5-hydroxy-3,4",6,7-tetramethoxyflavone **4**, 5,4'-dihydroxy-3,6,7-trimethoxyflavone **5**, 5,7,8,4'-tetra hydroxy-3,6-dimethoxy flavone **6**, l-C-syringylglycerol **7**, and fraxetin **8**. Their structural elucidation was performed using spectroscopic data (1D and 2D NMR) in combination with the literature values. The antibacterial, cytotoxic, and antioxidant activities of these isolated pure compounds were evaluated. Antibacterial assays, conducted against both Gram-positive and Gram-negative bacteria, revealed that the compounds exhibited no activity compared to standard antibiotics. In cytotoxicity tests using the KB-3-1 human cervix carcinoma cell line, the compounds **1, 3 and 5** showed moderate activity, with lower cytotoxicity relative to Cryptophycin 52. The antioxidant capacity, assessed using the DPPH radical scavenging assay, and compounds **1** and **3** indicated weak free radical scavenging potential. These findings highlight the chemical diversity of *Dodonaea angustifolia* twigs and their potential as sources of bioactive compounds for medicinal applications.

KEY WORDS: *Dodonaea angustifolia*, Structural elucidation, Cytotoxicity test, Antibacterial activity, Antioxidant activity

INTRODUCTION

Dodonaea, commonly known as hop-bush or sand olive, is a genus consisting of approximately 70 species [1]. One of its species, *Dodonaea angustifolia* L.f., belongs to the Sapindaceae family and is a medium-sized shrub or small tree, growing between 0.5 to 7.5 meters tall. Its glossy green leaves are coated with a sticky secretion, with newer leaves being stickier than older ones, which have a distinct rough, sandpaper-like texture. *Dodonaea angustifolia* is highly variable across its wide natural distribution, spanning regions in Australia, Africa, Asia, and South America. Numerous unique populations have been classified as separate species [2]. In Ethiopia, it is known by several local names such as Karkare (Agew), Kitkitta (Amharic), Termien (Ge'ez), Ettecca (Oromiffa), Intanca (Sidama), Tahses (Tigrigna), and Den (Somali) [3]. Traditionally, this plant has been used to treat various conditions, including tuberculosis and pneumonia [4], and is also known for its antibacterial [5], antifungal [6], antidiabetic [7], anti-inflammatory [8], antidiarrheal [9], and antiplasmodial properties [10]. Reports of phytochemical work on *Dodonaea angustifolia* which is found in Ethiopia are limited, with only a few secondary metabolites having been reported from different parts of the plant. Herein we report chemical constituents of the twigs of *Dodonaea angustifolia* and their bioactivity tests such as antibacterial, cytotoxicity and radical scavenging activities.

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RESULTS AND DISCUSSION

Reports of phytochemical work on *Dodonaea angustifolia* which is found in Ethiopia are limited, with only a few secondary metabolites having been reported from different parts of the plant. Based on this, our investigation has been done on the twigs of *Dodonaea angustifolia* and resulted on isolation of eight (8) pure compounds. Their structures were determined using 1D and 2D NMR spectra in combination with the literature values. As an example, compound **5** from the Figure 1 has been chosen and its NMR data will be discussed compared to the literature value (Table 1) as follows. Compound **5** was obtained as yellow solid and has a total of 18-carbons with a molecular formula $C_{18}H_{16}O_7$ and the molecular mass of the compound is 344 [11]. According to 1D (^{1}H, ^{13}C and DEPT NMR) and 2D (COSY, HSQC and HMBC NMR) spectra and the literature values, the compound has 10 quaternary carbons and 8 protonated ones. The singlet peak at δ_H 12.77 ppm is an evidence for the presence of the chelated proton at position C-5 of the ring A of flavonoid skeleton. The presence of a singlet proton peak at δ_H 6.82 ppm (at δ_C 90.8 ppm) which is more shielded showed about the presence of the substitution at positions 6 and 7 of the ring A of flavonoid skeleton besides position 5. The HMBC spectrum confirmed the presence of methoxy groups at the positions 6 and 7 of the ring A of the flavonoid skeleton. In addition, the presence of one of the methoxy group at position 3 of ring C of the flavonoid skeleton was confirmed by using HMBC correlations of 2D spectrum of the compound. Furthermore, the aromatic region of the ^{1}H NMR spectrum for ring B of the flavonoid skeleton for compound **5** showed two sets of the ortho-coupled proton signals at δ_H 8.06 (2H, d, J = 10.0 Hz, H-2'/6') and 7.03 (2H, d, J = 10.0 Hz, H-3'/5') and presented the presence of a 1,4-disubstituted aromatic ring with an AA'BB' system [12]. Based on the information discussed above and with comparison of the literature value, compound **5** was characterized and named as 5,4'-dihydroxy-3,6,7-trimethoxyflavone, and is also called penduletin [11]. This compound was isolated and reported for the first time in 1958 in the glucosylated form, from the ethanolic extract of *Brickellia pendula* obtained in Contreras near Mexico City [13]. It was also isolated and reported from the extract of the surface exudates of the leaves of *Dodonaea angustifolia* [14] and *Dodonaea viscosa* [15]. The other known compounds that have been isolated and reported herein from the twigs of the *Dodonaea angustifolia* includes 5,7-dihydro-3,4′,6-trimethoxyflavone and also called santin **1** [10, 14], 5,6,7-trihydroxy-3,4'-dimethoxyflavone **2** [10], 5,7,4'-trihydroxy-3,6-dimethoxyflavone **3** [10], 5-hydroxy-3,4",6,7-tetramethoxyflavone **4** [16], 5,7,8,4'-tetra hydroxy-3,6-dimethoxyflavone **6** [17], l-*C*-syringylglycerol **7** [18], and fraxetin **8** [19]. All the isolated compounds reported for the first time from the twigs of *D. angustifolia*. The cytotoxicity, antibacterial and antioxidant activity of some of the isolated compounds were tested.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR chemical shifts for compound **5** compared to the literatures values in acetone-d_6.

Position	δ_H (ppm)		δ_C (ppm)	
	Compound **5**	Lit. value [11]	Compound **5**	Lit. value [11]
2	-	-	159.2	158.6
3	-	-	138.2	139.4
4	-	-	178.9	180.4
5	-	-	152.8	
6	-	-	132.2	133.5
7	-	-	160.2	160.5
8	6.82, *s*	6.76, s	90.8	92.5
9	-	-	152.2	154.1
10	-	-	106.2	107.1
1'	-	-	121.5	122.5
2'	8.06, *d* (10.0 Hz)	8.02, *d* (8.7 Hz)	130.3	131.5

3'	7.03, *d* (10.0 Hz)	6.95, *d* (8.7 Hz)	115.3	116.7
4'	-	-	156.2	161.5
5'	7.03, *d* (10.0 Hz)	6.95, *d* (8.7 Hz)	115.2	116.7
6'	8.06, *d* (10.0 Hz)	8.02, *d* (8.7 Hz)	130.3	131.5
3-OMe	3.88, *s*	3.78, *s*	59.3	60.7
6-OMe	3.81, *s*	3.83, *s*	59.8	61.3
7-OMe	3.99, *s*	3.96, *s*	55.8	57.1
5-OH	12.77, *s*		-	-

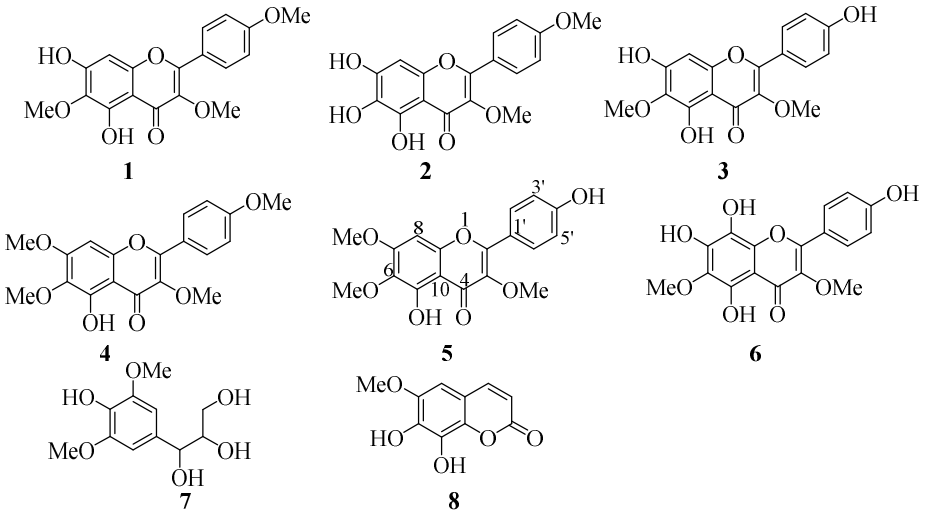

Figure 1. Chemical structures of compounds isolated from twigs of *D. angustifolia*.

Cytotoxicity assays

Three pure compounds were tested for cytotoxicity against KB-3-1 human cervix carcinoma cells using a cell-based resazurin assay with fluorescence detection. This method measures the reduction of resazurin to resorufin as an indicator of cell viability [20]. As summarized in Table 2, the three pure compounds exhibited moderate cytotoxicity compared to the standard drug Griseofulvin. However, their cytotoxic effects were negligible when compared to the potent standard, Cryptophycin 52. Among the three pure compounds, compound **1** showed relatively higher cytotoxic activity compared to the other two pure compounds. In general, the activities of the compounds were found in the order of: **1** > **3** > **5** in cytotoxicity.

Table 2. Cytotoxicity data for pure compounds from the twigs of *Dodonaea angustifolia*.

Compounds	Concentration (mol/L)	IC_{50}	95% Confidence intervals
1	0.1	9.63 µM	6.315 to 14.69 µM
3	0.1	18.7 µM	12.67 to 27.70 µM
5	0.1	23.6 µM	20.90 to 26.60 µM
Cryptophycin 52	0.1	13 pM	11-18 pM
Griseofulvin	0.1	19.3 µM	6 µg/mL to 7.4 µg/mL

Key: µM = micro molar; pM = pico molar.

Antibacterial test

The *in vitro* antibacterial activity of three pure compounds isolated from the twigs of *Dodonaea angustifolia* was assessed using the agar well diffusion assay, as shown in Table 3. Non-pathogenic model organisms, sourced from the Leibniz Institute of Germany–DSMZ (German Collection of Microorganisms and Cell Cultures GmbH), were used in this assay. The test panel included Gram-positive bacteria such as *Bacillus subtilis* (DSMZ 704), *Micrococcus luteus* (DSMZ 1605), and *Staphylococcus warneri* (DSMZ 20036), as well as Gram-negative bacteria like *Escherichia coli* (DSMZ 1058) and *Pseudomonas agarici* (DSMZ 11810). The results indicated that none of the tested pure compounds exhibited antibacterial activity against the organisms, as no inhibition zones were observed (ZOI = 0 mm in diameter). In contrast, the standard antibiotic, gentamycin, demonstrated significant inhibition zones (ZOI = 16-22 mm in diameter) against both Gram-positive and Gram-negative bacteria.

Table 3. Antibacterial activity results for pure compounds isolated from the twigs of Dodonaea angustifolia using agar diffusion method compared to gentamycin standard.

Compounds	Concentration (mg/mL)	Gram negative bacteria		Gram positive bacteria		
		E. coli	*P. agarici*	*B. subtilis*	*M. luteus*	*S. warneri*
1	1	-	-	-	-	-
3	1	-	-	-	-	-
5	1	-	-	-	-	-
Gentamycin	0.5	+++	+++	+++	+++	+++

Key: - = not active, +++ = strongly active, Gentamycin = standard.

Antioxidant activity

As shown in the Table 4, the compounds **1** and **3** showed very weak antioxidant activities with IC_{50} values above 250 and 200 µg/mL respectively with the same concentration range to that of Trolox which was used as standard during this study. However, the compound **5** does not show any activity with the same concentration to that of the standard and the other two compounds.

Table 4. Antioxidant results for pure compounds isolated from the twigs of *Dodonaea angustifolia* compared to reference Trolox.

Compound	Concentration range (µg/mL)	IC_{50} (µg/mL)
1	500-0.25	>200
3	500-0.25	>250
5	500-0.25	Not active
Trolox- Reference	500-0.25	11.04

EXPERIMENTAL

General

^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC and HMBC of the compounds were recorded using Bruker Avance DRX 500 and DRX 600 MHz spectrometers using standard pulse sequences and referenced to residual solvent signals. Column chromatography was carried out on silica gel 60 (0.040–0.063 mm, Merck). Preparative TLC (0.5 mm thick) and analytical TLC were performed with precoated Merck silica gel 60 $PF_{254+366}$. Visualisation of chromatograms were done under UV light (254 and 366 nm) and further by spraying 10% sulphuric acid followed by heating.

Plant materials

The fresh twigs of *Dodonaea angustifolia* were collected in February 2024 from Quarite Woreda, which is located approximately 400 km northwest of Addis Ababa. This area is situated in the West Gojjam administrative zone of the Amhara Regional State, about 250 km from Bahir Dar, the capital city of the region. The plant material was identified and authenticated by a botanist from the Department of Biology at Bahir Dar University.

Extraction and isolation

Five hundred grams of air-dried, ground fresh twigs of *Dodonaea angustifolia* were extracted with a 1:1 dichloromethane and methanol mixture, resulting in 20 g of a black gummy extract. This extract (20 g) was then adsorbed onto 100 g of silica gel and subjected to column chromatography using 100% cyclohexane as the packing solvent. The column was eluted with solvents of increasing polarity, first ethyl acetate, followed by methanol, yielding 22 fractions of 250 mL each. Based on thin-layer chromatography (TLC) analysis, the 22 fractions were combined into five main groups, labelled as A (1-5), B (6-10), C (11-13), D (14-18), and E (19-22).

Fraction C (11-13) (2 g) from the main fractions was subjected to column chromatography, packed with 100% cyclohexane, and eluted with solvents of increasing polarity using ethyl acetate, yielding approximately 15 sub-fractions, each 50 mL. Sub fraction 6 was further processed using preparative thin layer chromatography (PTLC) and eluted with a 9:1 mixture of cyclohexane and ethyl acetate (3 developments). This process, involving multiple developments, resulted in the isolation of a pure compound **1** weighing 26 mg. Sub fractions 7 and 8 were also combined together (200 mg) and subjected to column chromatography packed with 100% cyclohexane, and eluted with solvents of increasing polarity using ethyl acetate, yielding 6 sub fractions (1-6) each 10 mL. Among these 6 sub fractions, fractions 3-5 were combined together and subjected to preparative thin layer chromatography (PTLC), and eluted with a 9:1 cyclohexane and ethyl acetate mixture (3 developments). This multiple development process produced one pure compound **1** weighing (12.9 mg), which is identical to the compound isolated from sub fraction 6 above. Additionally, sub fractions 9, 10, and 11 were combined together (100 mg) and subjected to column chromatography packed with 100% cyclohexane, and eluted with the solvents of increasing polarity using ethyl acetate, yielding 5 sub fractions (1-5) each 10 mL. Among these five fractions, fraction 3 was subjected to preparative thin layer chromatography (PTLC), and eluted with a 9:1 cyclohexane and ethyl acetate mixture (3 developments). This multiple development process produced one pure compound **2** of 1.5 mg.

Among five main fractions, sub fractions D (14-18) (3 g) were subjected to column chromatography, packed with 100% cyclohexane, and eluted with increasing polarity using ethyl acetate, yielding 20 sub fractions each with 50 mL. Based on TLC analysis, the fractions were grouped and recombined in to a, b, c and d. Sub fractions 5 and 6 (labelled as sub fraction b) were further purified using preparative thin layer chromatography (PTLC) and eluted with a 4:1 cyclohexane and ethyl acetate solvent system, resulting in two pure compounds **3** and **4** weighing 5.6 mg and 1.4 mg, respectively, with multiple developments. Additionally, sub fractions 9 and 10 (labelled as sub fraction d) were combined together and subjected to column chromatography, packed with 100% cyclohexane, and eluted with ethyl acetate of increasing polarity, producing six sub fractions (d1–d6) each with 50 mL. Among these, sub fraction d2 was further processed using preparative thin layer chromatography (PTLC), eluted with a 7:3 cyclohexane and ethyl acetate mixture, and yielded a pure compound **5** weighing 25 mg with multiple developments. Among those four sub fractions, sub fractions 7 and 8 were combined together and subjected to column chromatography, which was packed with 100% cyclohexane, and eluted with increasing polarity using ethyl acetate. This process yielded 10 sub fractions (labelled a–j). Among them,

sub fractions f and g were combined together and further purified using preparative thin layer chromatography (PTLC) with a 4:1 cyclohexane and ethyl acetate solvent system, involving multiple developments, which resulted in the isolation of one pure compound **6** weighing 1.5 mg. Similarly, sub fractions i and j were combined together and applied to preparative thin layer chromatography (PTLC), and after multiple developments, yielded mixture of pure compounds **7** and **8** weighing 3.2 mg.

Cytotoxicity assays

The cytotoxic activity of the isolates from the twigs of *Dodonaea angustifolia* was assessed as previously described before [20]. Briefly, KB-3-1 cells were cultured as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose, L-glutamine, sodium pyruvate, and phenol red, supplemented with 10% foetal bovine serum (FBS). The cells were maintained at 37 °C in a humidified atmosphere with 5.3% CO_2. On the day before testing, cells at 70% confluence were detached using a trypsin-ethylenediamine tetraacetic acid (EDTA) solution (0.05%; 0.02% in Dulbecco's Phosphate Buffered Saline, DPBS) and seeded into sterile 96-well plates at a density of 1000 cells per well in 100 µL of medium. Dilution series of the test compounds were prepared from stock solutions in DMSO (at concentrations of 100 mM, 50 mM, or 25 mM) and further diluted with culture medium (containing 10% FBS) to reach concentrations in the pM range. These dilutions were added to the wells, with each concentration tested in six replicates. Serial dilutions were made by transferring liquid from one well to the next. Controls contained the same DMSO concentration as the first dilution. After 72 hours of incubation at 37 °C in 5.3% CO_2, 30 µL of an aqueous resazurin solution (175 µM) was added to each well. The cells were then incubated for an additional 5 hours under the same conditions. Fluorescence was measured with excitation at 530 nm and emission at 588 nm. IC_{50} values, representing the concentration at which cell viability was reduced to 50%, were calculated using a sigmoidal dose-response curve in GRAPHPAD PRISM 4.03.

Antibacterial test

The antibacterial activity of pure isolates from the twigs of *Dodonaea angustifolia* was tested using the agar diffusion method as described by Sellem *et al.* [20, 21] in slight modification. In this approach, approximately 100 µL of a glycerine stock solution of the bacterial strain was added to 50 mL of Nutrient Broth No.1 (Sigma Aldrich, pH 7.0) or Trypticase Soy Broth (Sigma Aldrich, pH 7.0–7.2). The cultures were incubated overnight at around 150 rpm under the appropriate temperature conditions for each bacterial strain. Next, 0.2 mL of the overnight bacterial culture, with an optical density (OD) of 0.05–0.1, was plated onto a 90 mm petri dish (Sarstedt) containing either Nutrient Broth agar or Trypticase Soy Broth agar, depending on the bacteria. A sterile paper disk (6 mm diameter, Carl Roth) was then placed on the bacterial plate using sterile forceps. Each disk was loaded with 25 µL of a drug solution, either 40 mg/mL of the plant extract in DMSO or 10 mM of the pure compound in DMSO, diluted to 1.0 mg/mL with media. For reference, 25 µL of Gentamycin (0.5 mg/mL, 5% DMSO in media) was used, and DMSO alone served as the negative control. After 24 hours of incubation at 37 °C or 30 °C (depending on the bacterial strain), inhibition zones formed around the paper disks. These zones were measured in millimetres two or three times, and the average was calculated. An absence of an inhibition zone indicated no activity, while a zone measuring 6.5 to 8 mm indicated intermediate activity. Zones greater than 9 or 10 mm indicated significant antibacterial activity, and further testing for the minimum inhibitory concentration (MIC) could be conducted.

Antioxidant activity

Antioxidant activity of pure compounds from the twigs of *Dodonaea angustifolia* was tested by using DPPH as descried by Sellem *et al.* [21] in slight modification. In this approach, pure compounds were dissolved in ethanol (EtOH p.A.) or methanol (MeOH p.A.) and diluted at different concentrations (500, 250, 125, 60.25, 30.125 µg/mL, etc.). Then, 50 µL of 0.2 mg/mL solution of DPPH radical in ethanol was mixed with 50 µL of samples. The mixture was incubated for 30 min in the dark at room temperature. The scavenging capacity was determined spectrophotometrically by monitoring the decrease in absorbance at 517 nm against a blank. The percentage of antiradical activity (%ArA) had been calculated as follows:

%ArA = [(absorbance of control - absorbance of test sample)/absorbance of control] x 100

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