Isolation and Structural Elucidation of Secondary Metabolites from *Dodonaea viscosa* and Qualitative Screening of its Sub Fractions Based on GC-MS

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Summary: A phytochemical investigation of *Dodonaea viscosa* plant extract was conducted. A new 3,5,7-trihydroxy-6,4′-dimethoxy-3′-isoprenyl-flavone (**1**) and three known compounds Hautriwaic lactone (**2**), Kamempferol 3-methyl ether (**3**) and vanillic acid (**4**) were isolated from the aerial parts of *Dodonaea viscosa*. The structure of the isolated constituents was elucidated using 1D and 2D Nuclear Magnetic Resonance (NMR) and mass spectrometry. The qualitative study of the Fraction 1 was performed by using Gas Chromatography (GC) and Gas Chromatography – Mass Spectrometry (GC-MS). The nature of compounds was confirmed by NIST library search. The antimicrobial activity and possible use of the plant extracts were also elaborated.

Keywords: *Dodonaea viscosa***,** GC-MS analysis, Antimicrobial activity, Phytochemical investigation, Minimum Bactericidal concentration

Introduction

Dodonaeaviscosa is a perennial herb native to the Southern hemisphere and grows in South East Asia including India, Pakistan, China, Australia, South Africa, New Zealand, Latin America and some other parts of the world [1]. The plant is named after a Flemish botanist, royal physician and professor of 16th century Rembert Dodoens. The plant is a part of scrub vegetation mostly present in the low hilly areas. The plant is also used as fire-wood and commonly used for making mud structures in villages and also walking sticks and handles are made from the wood.

Dodonaea viscosa is proven to have many medicinal properties the bark of the plant is administered orally or as poultice to treat swellings, fever and headache. The extracts of stem and leaves are used to treat sore throats, while the infusion of roots is used to treat cold while the seed combinations are used for malaria. The stems of the plant are used for fumigation and also for the treatment of rheumatism. The leaves and roots are used as an antispasmodic agent (treating digestive system disorders) and also relieve itching, fever swelling and aches [2, 3]. A topical lotion made from different parts of the plant is used to treat sprains, bruises, burns and wounds.

The leaf juice and crushed roots of the plant are mostly the part of anthelmintic preparations and used for expulsion of the roundworms. The plant roots are also effective to stimulate postpartum milk production and for the treatment of irregular menstruation and dysmenorrhoea. *Dodonaea viscosa's* flower extract is also used as a tonic [4]. In some areas of South East Asia the seeds are used as fish poison [5]. The leaves are found to have antifungal, antidiabetic, antibacterial, anti-inflammatory and antioxidant properties [6].

The plant contains flavonoids, tannins, saponins, steroids and triterpenes with varying activities. The chemical studies of *Dodonaea viscosa* has resulted in the identification of various flavonoids [7], the diterpenoid acids [8, 9], number of biologically active saponins [9, 10], plant acids [5, 10], *p*-coumarin acid ester-18, number of essential oils [11], sterols [8, 12]and tannins [10] from the aerial parts of the plant, while saponins esters were extracted from the seeds of *Dodonaea viscosa* [5, 13].

Some of the studies demonstrated the presence of 42 different flavonoids from leaves, bark, flowers and seeds of the plant, including Rutin (quercetin glycoside) and Isorhamentin [14, 15]. Large concentrations of Quercetin, Kaempferol and Isorhamnetin were isolated more recently from the leaf extract of the plant [16].

Experimental

Material and Methods

General Experimental Procedure

Ultraviolet spectra were performed using a Hitachi-U-3200 spectrophotometer, while the IR

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spectra were performed using a JASCO-A-302 spectrophotometer. A Finnigan Mat-311 A, spectrometer was used to record the mass spectra at the temperature of 250°C and electron energy of 70eV. Bruker AV-300 and Bruker AM 400 spectrometers were used to record both 1D and 2D nuclear magnetic resonance spectra in acetone, operating at 500 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR. The column chromatography was conducted using Hz. Silica gel-60 (Merck, kieselgel-60, 70-230 mesh size) to record the chemical shifts in ppm (δ) and coupling constants (*J*). Thin Layer Chromatography (TLC) was performed using silica gel 60 (PF_{245}; Merck) coated on glass plates and the analytical chromatographwas obtained using Aluminum cards precoated (0.5 mm thickness) with silica gel (SiF_{254}; Merck), while silica gel 60 (GF_{245}; Merck) was used for vacuumliquid chromatography (VLC).

Description of material

The plant material was obtained from the mountainous region of KPK, Pakistan. The identification was performed at the Department of Botany, Post Graduate College Kohat by a plant taxonomist, Dr. I Khan. A specimen from the collected plant was deposited to the Department of Botany, Post Graduate College Kohat with the voucher/specimen number DVPGCK - 098.

Extraction and Isolation

The extraction was carried out with methanol (MeOH; 30Lx3x10 days) at room temperature from a powdered specimen of 20 Kg shade-dried plant material. The extracts were then concentrated under reduced pressure yielding 2 Kg of a gummy brown residue. The resulting extract was suspended in 2 L of water and successive extractions were carried out with *n*-hexane (5L×3), ethyl acetate (5L×3), methanol (5L×3), *n*-butanol (5L×3) and water to yield fractions soluble in *n*-hexane (610 g), ethyl acetate (506 g), methanol (390 g), *n*-butanol (700 g) and water respectively. The fraction soluble in ethyl acetate (EtOAc) was passed through Vaccum Liquid Chromatography (VLC) on a silica gel and further eluted with *n*-hexane-$CHCl_3$, $CHCl_3$-EtOAc, EtOAc-MeOH, and MeOH with gradient increase of polarity, yielding 38 fractions. Due to the oily nature of Fraction-1, it was submitted for GC GC-MS analysis. Fraction-7 (1.2 g) resulted in 3,5,7-trihydroxy-6,4′-dimethyoxy-3′-isoprenyl-flavone **1** (30 mg) by repeated silica gel column chromatography (CC) using *n*-hexane : EtOAc (8:2), *n*-hexane-$CHCl_3$ (1:1). Fraction-9 (980 mg) was subjected repeatedly to column chromatography (CC) using silica gel column and eluted with hexane-EtOAc (8:2) to yield Hautriwaic lactone **2** (40 mg), and Kamempferol 3-methyl ether **3** (25 mg). Fraction-10 (670 mg) was purified to vanillic acid **4** (18 mg) by using silica gel CC eluted with *n*-hexane-EtOAc (8:2). The isolated compounds passed the purity (> 99%), confirmed by TLC, NMR and electron ionization mass spectrometry (EI-MS).

Gas Chromatography (GC)

The qualitative analysis of the *Dodonaea viscosa* was performed with Gas Chromatography using flame ionization detector (FID) with a less polar capillary column OPTIMA®-5-Accent (60 m x 0.32 mm ID with film thickness 0.25µm of 5% Phenyl / 95% Methyl Silicone) installed on a Shimadzu GC-10. The initial temperature for the analysis was set at 50˚C for the duration of 2 minutes and was subsequently increased at the rate of 7˚C per minute, till the final temperature of 260˚C with FID at 400˚C. Nitrogen was used as the carrier and makeup gas having flow-rate of 12.334 mL/min at 90 kPapressure.

Gas Chromatography-Mass Spectrometry (GC-MS)

The Gas Chromatography-Mass Spectrometry was carried out by using Agilent 6890 gas Chromatograph, having ZB-5MS (30m x 0.32mm ID with 0.25µm film-thickness), the GC was attached to a Jeol JMS-600H mass spectrometer which was operating in Electron Ionization mode at 250˚C ion source and electron energy of 70eV. As tolerated by the detector response the volume of the carrier gas was adjusted between the ranges of 1.0 to 5.0 µL [17,18].

Screening of Antimicrobial activity

Agar Well Diffusion method was used for the screening of the antimicrobial activity of *Dodonaea viscosa* extracts. The plant extracts were test for antimicrobial activity against eleven Gram positive bacteria *(Bacillus subtilis, B.cereus, Staphylococcus aureus, S.saprophyticus, S.epidermidis, Enterococcus faecalis, Streptococcus pyogenes, Vancomycin Resistant Enterococci (VRE), Methicillin Resistant S.aureus (MRSA), Micrococcus luteus, Corynecacterium xerosis*), 9 Gram negative bacteria (*Salmonella typhi, Salmonella para typhi A, S. para typhi B Shigella* sp, *Klebsiella pneumoniae, Proteus mirabilis, Escherichia coli, , Enterobacter sp, Pseudomonas aeruginosa*), two yeast (*Candida albican and S accharomyces cerevisae*), two

dermatophytes (*Trichophyton mentagrophyte and Microsporum gypseum*) and seven saprophytes (*Aspergillus niger, Penicillium sp, Paccilomyces variola, Aspergillus flavus, Chrysporium sp, Fusarium oxysporum, Chrysporium sp, Aspergillus terreus, Aspergillus terricola).*

A Stock solution (20mg/mL) of plant extracts was prepared in sterilized dimethyl sulfoxide (DMSO) with DMSO as a negative control, and ciprofloxacin (CIP) as a positive control for bacteria, while Otosporin was used as a positive control for fungi.

Cell suspension was prepared by inoculation of 24 hours old bacterial culture and yeast in 5mL saline. Turbidity of cell suspension was matched with 0.5 McFarland standards, which is equivalent to $1.5x10^8$ CFU/mL microbialload. For fungal cultures, spore suspension ($5x10^5$ spores/mL) was made from 5 days old molds plates. Confluent lawn was made on Mueller-Hinton agar (MHA) plate and Sabourauds-Dextrose agar (SDA) for bacterial and fungal cultures, respectively, and allowed to dry for 5-10 minutes. The wells (8mm) were dug with the help of sterile borer. The extract solution (20µL) was introduced into the wells and allowed to diffuse into media for 15-20 minutes. The incubation of the plates was carried out at 37 ºC for 16-18 hours for bacteria and at ambient temperature for 5 days for fungi.

Determination of MIC and MBC

The Minimum Inhibitory Concentration (MIC) was determined for the extracts showing a significant zone of inhibition (>15) *i.e.* extracts giving >15mm of zone of inhibition on Agar well diffusion assay [19]. The extracts were dissolved in sterile DMSO with 20 mg/mL concentration. Sugar tubes with 1 mL of respected media broth i.e., Nutrient broth for bacterial and Sabourauds Dextrose broth for fungi was introduced with 1mL of extract solution to get final concentration of 10mg/mL and two fold serial dilution was done till 0.8 mg/mL of extracts concentration. Microbial suspension (50 µL) was added in each tube and incubated at aforementioned incubation conditions. Control tube contained media and microbial suspension only. The highest dilution that didn't show any visible turbidity was considered as MIC.

For determination of Minimum Bactericidal Concentration (MBC), 100 mL of broth was taken from the MIC tubes that didn't show any turbidity and inoculated on respected support media. The plates were incubated at appropriate incubation conditions and the lowest concentration of the extracts that didn't permit any visible colony formation after the period of incubation was considered as MBC.

3,5,7-trihydroxy-6,4'-dimethoxy-3'-isoprenyl-flavone **(1)**

Yellowish green ($CHCl_3$), 10 mg, IR spectrum (KBr, υ_{max}, cm^{-1}): 3417(OH), 2918 (CH), 1657(C=O), 1611-1420 (Ar), UV spectrum ($CHCl_3$, λ_{max}, nm) 252, 270 and 350. ^1H and ^{13}C NMR data, Table-1. Mass spectrum EI-MS, *m/z* 398, HREI-MS, *m/z* 398.6410 (calcd. 398.1365). EI-MS *m/z* 69, 175, 182 and 216.

Table-1: ^{13}C (150 MHz) and ^1H (400 MHz) NMR Data of 1 (acetone, δ, ppm, J/Hz).

Position	^{13}C	^1H (*J* in Hz)
2	158.5	-
3	138.0	-
4	179.8	-
5	154.2	-
6	130.1	-
7	157.3	-
8	94.4	6.52 (s)
9	155.0	-
10	106.1	-
1'	123.4	-
2'	130.7	7.94 d (*J* =2.2)
3'	127.2	-
4'	160.5	-
5'	115.7	7.01 (*J* = 8.5, d)
6'	128.3	7.84 (*J* = 2.2, 8.5, dd)
1"	28.7	3.39 (d)
2"	122.9	5.41 (t)
3"	151.7	-
4"	17.7	1.75 (s)
5"	25.7	1.78 (s)
OCH_3(C6)	60.4	3. 85 (s)
OCH_3(C4′)	59.9	3.84 (s)

Result and Discussion

Compound **1** was isolated as yellowish green in colour with a molecular formula of $C_{22}H_{22}O_7$. The EI-MS showed molecular ion at *m/z* 398 and HREIMS at *m/z* 398.1310 (calcd. 398.1365), showed ions at *m/z* 69, 175, 182 and 216. The HR-EI-MS of these ions corresponded to the elemental compositions $(C_5H_9)^+$, $(C_{12}H_{15}O)^+$, $(C_8H_6O_5)$ and $(C_{14}H_{16}O_2)$ respectively illustrated in Fig. 1. The IR spectrum (KBr, υ_{max}, cm^{-1}) suggested the presence of hydroxy (3417 cm^{-1}) and conjugated carbonyl (1657 cm^{-1}) group in **1**. The UV spectrum ($CHCl_3$) of **1** showed λ_{max} at 252, 270 and 350 nm indicated the presence of flavone nucleus. The ^1H NMR spectroscopic data, with 10 degree of unsaturation, (Table 1) show an aromatic proton at 6.52 H-8(1H, s), three *ortho* and *meta*- coupled aromatic protons at $\&_H$7.94 H-2′ (1H,d, *J* =2.2), 7.01 H-5′ (1H, d, *J* =8.5) and 7.84 H-6′ (1H, dd, *J* = 2.2, 8.5).and two methoxy groups at $\&_H$ 3.85 (s) and 3.84 (s). The NMR data

also demonstrated the presence of 3-methyl-2-butenyl group &$_H$ 1.75 and 1.78 (each 3H, s), 5.41 (1H, t, *J* =7.00). The ^{13}C NMR showed 22 carbon signals of flavonol derivative along with two methoxy &C 60.4 and 59.9, two methyl, one methylene, five methine and twelve quaternary carbons. The position of 3-methyl-2-butenyl group was further supported by the HMBC correlation (Fig 1) from H-1″ to C-2′, C-3′, C-2″, and C-3″. The heteronuclecular multiple bond correlation (HMBC) was showed correlations between H-8 and C-7, C-9 and C10, H-5′ and C-6′, C-4′ and H-2′ and C-1″ [20] and the other correlations are also elaborated in the Fig 2.

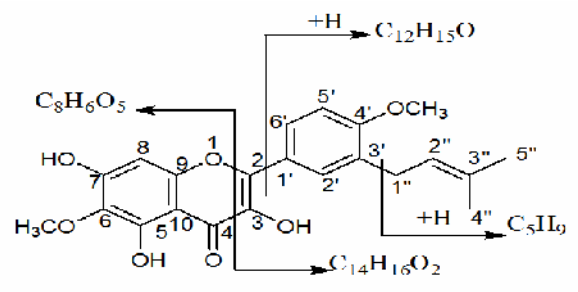

Fig. 1: HR-EI-MS of **1**.

The GC-MS chromatogram and chemical composition of fraction 1 identified in *Dodonea viscosa* are reported in Fig 3 and Table-2 respectively. A total of 24 compounds were identified during the study, major compounds are Aromadendrene oxide-(1) (53.57%) and Asarone (17.55%). Aromadendrene oxide-(1) is a Sesquiterpene oxide which are known to have different activities like anti-tumor, analgesic, antibacterial, antiinflammatory, sedative, fungicide [21] and the oxygenated sesquiterpenes and flavonoids have more antioxidant activity than the non-oxygenated sesquiterpenes [22, 23].

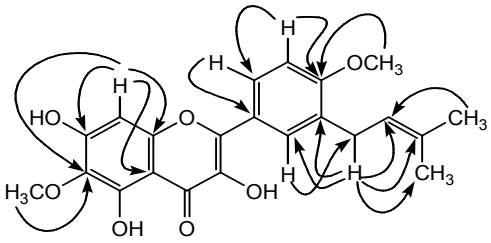

Fig. 2: KeyHMBC correlations of **1.**

The present study evaluated the antibacterial activity *n*-hexane, chloroform, ethyl acetate, *n*-butanol extract (Table 3). The extracts showed strong activity against Gram positive bacteria including *VRE, S.pyogenes, S.aureus, B.cereus, C.xerosis* and yeast. No zone of inhibition was observed against remaining Gram positive culture, all Gram negative bacteria and molds. Extracts that were showing potent antimicrobial activity was further examined for their MIC. Most of the extracts showed MIC ranged from 1.25-0.75 mg/mL for *VRE, S.pyogenes, S.aureus, B.cereus, C.xerosis* while MIC ranged was 5-0.75 mg/mL for the yeast cultures.

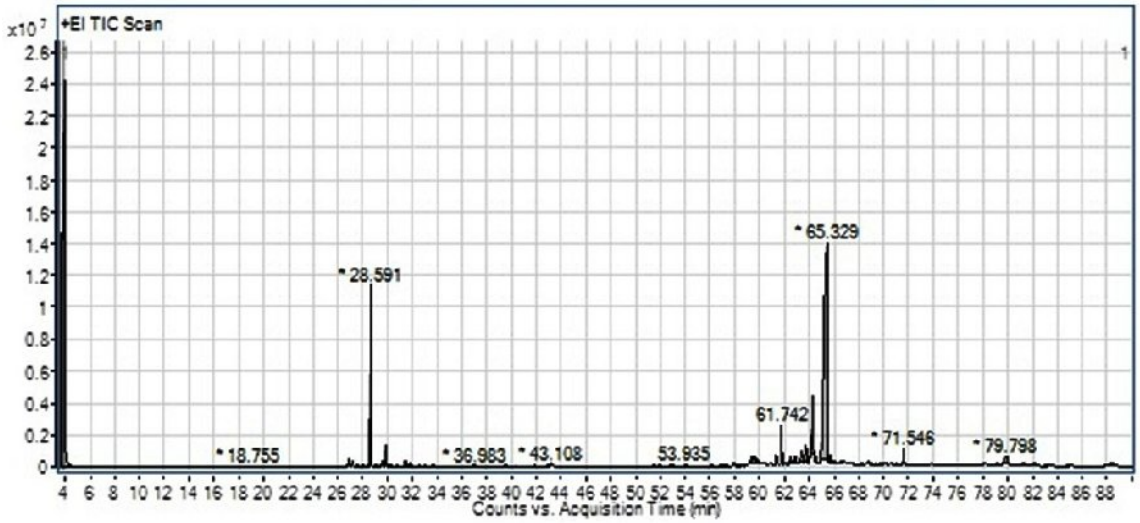

Fig. 3: GC-MS Chromatograms fraction 1 of *Dodonaeaviscose.*

Table-2: Relative concentrations and list of the compounds present in the fraction 1.

Peak No	RT* (mins)	RI*	Relative % Conc.	Compounds Name
1	26.859	1279	0.54	2,2'-Isopropylidenedifuran
2	27.094	1426	0.26	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-
3	27.543	1530	0.09	4aH-Cycloprop[e]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl-, [1aR-(1aα,4β,4aβ,7α,7aβ,7bα)]
4	28.591	1568	17.55	Asarone
5	29.475	1593	0.77	2-Naphthalenemethanol, decahydro-α,α,4a-trimethyl-8-methylene-, [2R-(2α,4aα,8aβ)]
6	29.740	1673	1.33	Acetic acid, 1-methyl-3-(2,2,6-trimethyl-bicyclo[4.1.0]hept-1-yl)-propenyl ester
7	31.364	1558	0.55	1,2-Dimethoxy-4-(1,2-dimethoxyethyl)benzene
8	31.766	1838	0.47	Cycloheptanone, 4-acetyl-7,7-dimethyl-2-(2-oxopropyl)
9	33.681	2045	0.14	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
10	59.491	2030	2.26	5,8,11-Heptadecatriynoic acid, methyl ester
11	61.293	2517	0.81	Pregn-5-en-20-one, 3-(acetyloxy)-16,17-epoxy-6-methyl-, (3β,16α)-
12	61.742	2186	3.05	Norethindrone
13	62.365	2112	0.40	Pregnan-20-one, 3,5-dihydroxy-6-methyl-, (3 β,5 α,6 β)-
14	62.777	3942	0.52	1-Heptatriacotanol
15	63.310	2820	0.95	Oleic Acid
16	63.323	2082	1.77	9-Octadecenoic acid (Z)-, methyl ester
17	64.234	2822	9.78	Resibufogenin
18	64.428	2896	0.14	Ursodeoxycholic acid
19	65.329	1462	53.57	Aromadendrene oxide-(1)
20	65.440	2774	0.29	Phorbol
21	65.587	2595	0.35	Pregn-5-ene-3,20-diol-13-carboxylic acid, 3-acetate-18,20-lactone
22	68.679	2440	0.29	Pregnan-20-one, 3-(acetyloxy)-5,6:16,17-diepoxy-, (3β,5α,6α,16α)-
23	71.546	3149	1.77	Vitamin E
24	79.798	2848	2.39	Lupeol
			100.04	

*RT= Retention time, *RI= Refractive index

Table-3: MIC and ZOI of extracts.

Extracts	*B.cereus*		*VRE*		*S.pyogenes*		*C.xerosis*		*E.fecalis*	
	MIC mg/mL	ZOI mm	MIC mg/mL	ZOI mm	MIC mg/mL	ZOI mm	MIC mg/mL	ZOI mm	MIC mg/mL	ZOI mm
n-hexane	-	-	-	-	-	-	2.5	17	5	15
Ethyl acetate	0.75	14	1.25	17	2.5	14	10	15	2.5	15
Methanol	-	-	-	-	-	-	-	-	10	20
n- Butanol	-	-	-	-	-	-	0.75	15	10	13
Water	-	-	-	-	-	-	-	-	-	-
Fraction 1	-	-	2.5	17	5	20	1.25	15	1.25	14

Conclusion

The chemical composition of *n*-hexane extract identified in *Dodonea viscosa* was reported and a total 24 compounds identified. The major compounds identified in the study are Aromadendrene oxide-(1) and Asarone The plant extracts showed strong activity against selected Gram positive bacteria and yeast, while no activity against Gram negative bacteria mold`. The MICs of the extracts were also determined.

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