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Volatile Composition and Antibacterial Activity of Fruits of *Withania coagulans* Dunal

Iqra Nazir[a], Azeem Intisar[a]*, Muhammad Imran Din[a], Jan Sher Khan[b], Ejaz Ahmed[a], Aqsa Aamir[a], Mujahid Manzoor[c] and Muhammad Irfan[d]

[a]School of Chemistry, University of the Punjab, Lahore, Pakistan

[b]Department of Botany, University of the Punjab, Lahore, Pakistan

[c]Faculty of Agricultural Sciences, University of the Punjab, Lahore, Pakistan

[d]Department of Biomedical Physics, Doctoral School of Exact Sciences, Adam Mickiewicz University Poznan, Poland

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Abstract. *Withania coagulans* Dunal is a significant medicinal plant as its fruit is used to treat numerous ailments. In this study, volatile oil of fruit of *Withania coagulans* was extracted by microwave associated hydro-distillation and analyzed by gas chromatography mass spectrometry that resulted in the successful identification of 40 constituents where the most dominating ones were: caprylic acid (28.58%), caproic acid (16.88%), capric acid (12.21%), lauric acid (5.77%), palmitic acid (3.49%), eugenol (3.96%) and varamol (2.95%). The fruit was found to be a major source of fatty acids (71.91%). Moreover, the anti-bacterial activity of the oil against various drug-resistant microbes demonstrated its potential against *Pseudomonas aeruginosa* (21 mm), *Enterococcus faecalis* (11 mm) and *Methicillin* resistant *Staphylococcus aureus* (10 mm) at a dose level of 0.48 mg. Most of these constituents are well known bioactive agents. These results demonstrate the potential of this oil as a natural source of obtaining various fatty acids and a possible remedy against various microbial diseases.

Keywords: *Withania coagulans*, dodi paneer, volatile oil, fatty acids, drug-resistant bacteria

Introduction

Withania coagulans Dunal (Dodi paneer) belongs to family Solanaceae. It is a small whitish-gray shrub found in various regions of Pakistan and India. Fruits of the plant are brown in colour and sweet in taste (Gupta *et al*., 2021). It is generally known as the 'Indian Cheese-Maker' or 'Vegetable Rennet' as it contains an important enzyme (withanin) which is used to coagulate milk to cheese (Ahmadi *et al*., 2021; Salehi *et al*., 2017; Rahman *et al*., 2003).

Chemical investigations have revealed that it contains alkaloids, with anolides, lignans, steroids, tannins, carbohydrates, saponins, fatty acids, amino acids, organic acids and volatile oils (Gupta *et al*., 2021; Ali *et al*., 2017; Shahnaz *et al*., 2010; Alam *et al*., 2003; Rehman *et al*., 1998; Choudhary *et al*., 1995). The fruit oil of this plant exhibits fatty acids (saturated and unsaturated) as the most abundant class of compounds (Ali *et al*., 2017) which possesses dietary and pharmacologically important compounds such as lauric, linoleic, oleic, stearic, palmitoleic and palmitic acids etc. (Gupta *et al*., 2021; Yang *et al*., 2011).

*Author for correspondence;
E-mail: azeemintisar.chem@pu.edu.pk

This plant has been used as a significant medicinal plant for the treatment of numerous diseases. Its fruit is recognized as 'the magic healer' (Atarod *et al*., 2016) that exhibits sedative, emetic and diuretic properties. Dry fruits are administered as dietary supplements for the treatment of diabetes (Meeran *et al*., 2020). More-over, it is also employed for the treatment of neuro-suppression, low blood glucose levels and heart problems (Maurya *et al*., 2010) and the whole plant is useful for asthma, chronic liver disorder, ulcers, rheumatism and blood cleaning (Tareen *et al*., 2010).

Pharmacologically, it exhibits hepatoprotective, immuno-modulatory, antidiabetic, antimicrobial, anti-inflam-matory, cytotoxic and antitumor characteristics (Grover *et al*., 2015; Mishra *et al*., 2013; Sudhanshu *et al*., 2012; Maurya *et al*., 2010). Lauric acid and palmitoleic acid possess antimicrobial properties (Ouattara *et al*., 1997). One of its important fatty acids, palmitoleic acid improves hypertriglyceridemia and hyperglycemia which increases insulin sensitivity, suppresses pro-inflammatory gene expressions and also regulates lipid metabolism. Besides, volatile oils of its fruits have been found active against *Vibrio cholerae*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and

Klebsiella pneumoniae (Ullah and Khan, 2018; Khan *et al*., 1993; Gaind and Budhiraja, 1967). Pathogenic microbes, especially the drug-resistant ones are a significant threat to life and various reports on essential oils have demonstrated their activity (Bojoviæ *et al*., 2018) which is attributed to their hydrophobic nature that imparts membrane permeabilizing ability towards bacterial cell wall (Lambert *et al*., 2001). This study was aimed to profile volatile ingredients and antibacterial activity of *W. coagulans* especially against drug-resistant microbes.

Materials and Methods

Microwave assisted oil extraction. Dried fruits (600 g) of 'Dodi paneer' in unprocessed form, which were purchased from Akbar Market Lahore, Pakistan and identified as *W. coagulans* Dunal by Dr. Farkhanda Jabeen of Department of Botany, University of the Punjab. Dried fruits were finely ground to fine powder where 200 g of plant material was subjected to a domestic microwave (Company: Nobel; Model # OM46SS) with an output voltage and frequency of 1000 W and 2450 MHz, respectively, associated steam distillation apparatus for 45 min at a temperature regulated thermostat at an adjusted output power of 60%. The extraction was carried out in triplicate for obtaining results. Oil was collected in an airtight and sealed vial and kept under –20 °C prior to its GCMS and antibacterial analyses.

Gas chromatography and mass spectrometry. Agilent GC 7890B equipped with MS5977A and used for the GCMS analysis and oil separation was carried out on a DB-5 MS column (dimensions: 30 m × 250 µm ID × 0.25 µm thickness of film). Temperature ramp was employed that was initiated at 45 °C and given a hold for 3 min, late raised at 15 °C/min to 290 °C. Helium was employed as the mobile phase. The flow rate was maintained at 1 mL/min whereas injection volume of 1 µL dissolved oil was injected in split mode (split ratio 5:1). The ion source and transfer line temperatures were 250, 260 and 300 °C respectively. Mass to charge range (m/z) was selected between 35 to 500. Volatile ingredients were identified by comparing obtained spectra with standard mass spectra provided in NIST-11 library and their retention indices were also matched with literature (Adams, 2007; NIST mass spectral database 69). The relative percentage of oil constituents were calculated.

Antibacterial assay. The antibacterial activity was assessed *via* agar well diffusion method where three gram-negative (*P. aeruginosa*, *K. pneumoniae* and *E. coli*) and two gram-positive bacteria (*S. aureus and E. faecalis*) were evaluated in this study where noticeably *P. aeruginosa* was p5 strain, *S. aureus* was methicillin-resistant and all remaining strains were multidrug-resistant. These were provided by the Jinnah Hospital, Lahore and characterized by analytical profile index (API) test (Murray, 1978). Bacterial cultures were prepared and used within 24 h. In order to make the bacterial lawns for agar well diffusion, 1-3 colonies of the tested organism were picked (depending on their size) and spread on agar plates and left to dry for 5 min. Six holes (6 mm) were plunged into each plate where 4 wells were filled with dissolved volumes of known quantities (0.12 mg, 0.24 mg, 0.36 mg and 0.48 mg) and rest were introduced with negative (*n*-hexane) and positive controls (ampicillin 100 µg). Cultures were then incubated at 37 °C for 18 h and zones of inhibition were measured.

Results and Discussion

Volatile constituents of fruits of *W. coagulans*. Pale yellow oil was obtained from fruits of *W. coagulans* and its yield was 0.12%. The oil constituted 40 identified compounds. The constituents were arranged in the increasing order of their retention times as provided Table 1, where major volatiles were: caprylic acid (28.58%), caproic acid (16.88%), capric acid (12.21%), lauric acid (5.77%), palmitic acid (3.49%), eugenol (3.96%) and varamol (2.95%) and their mass spectra were shown in Fig. 1. The total oil percentage of all identified compounds was 94.55%. Most volatile constituents identified are well-known aromatic and fragrance producing ingredients that contribute to the aroma of its fruit. One of the well-known fragrant compounds is isoeugenol used in aerosols, cosmetics, soap and detergents, whereas phytol being profoundly available has extensive pharmacological importance (Sajjadi and Ghannadi, 2012).

Several components have already been extensively employed in medication as well as intensive care products such as camphor in vaporub inhalant that all these facts depict this oil as another natural warehouse of synergistically effective pharmaceutical constituents. Furthermore, carvone refrains premature budding of potato during storage and it is a well-known mosquito repellent (De Carvalho and Da Fonseca, 2006).

The one of the reported that the fruit oil of this plant (extracted by steam distillation), the total 20 compounds were identified which represented 90.80% total oil constituents (Shahnaz *et al*., 2010). However, the major portion was found to be sesquiterpenes (54%) and esters (21.50%), where the most abundant compound was caryophyllene (15.0%) whereas in our study, a total of 40 compounds were identified and the major classes were found to be fatty acids (71.91%) and oxygenated monoterpenes (7.87%) along with caprylic acid (28.58%) as the most abundant compound. However, various other fatty acids such as caprylic acid, capric acid, palmitic acid, linoleic acid and their esters were in qualitative concordance with other previous reports (Ali *et al*., 2017; Ali *et al*., 2015) with fatty acids being the major class of compounds. Besides, various other fatty acids such as lauric acid, myristic acid and stearic acid were also found.

Table 1. Volatile constituents of fruits of *W. coagulans*

Retention time (min)	Names of identified compounds	Retention index (RI_{Lit})	Relative abundance (%)
3.59	Dimethyl disulfide	747	0.52
4.23	3-Hexanone	781	t
4.47	Hexanal	801	0.28
4.72	1-Ethyl-1H-pyrrole	818	t
5.02	Furfural	828	0.75
5.95	2-Methyl butanoic acid	894	t
6.45	Hexanoic acid, methyl ester	921	0.22
7.01	5-Methyl-furfural	954	1.57
7.12	Dimethyl trisulfide	963	1.42
7.20	1-Octen-3-one	972	1.06
7.82	Caproic acid	1026	16.88
8.61	o-Guaiacol	1090	0.36
8.69	Undecane	1100	1.21
9.29	Camphor	1143	0.33
9.68	Caprylic acid	1167	28.58
9.81	Methyl salicylate	1182	0.29
10.00	(E)-2-Octenoic acid	1230	0.55
10.28	2-Methyl-3-phenyl-propanal	1244	0.83
10.30	Carvone	1239	t
10.42	Piperitone	1249	0.42
10.51	Nonanoic acid	1267	1.72
10.70	Thymol	1289	1.06
10.74	Tridecane	1300	0.45
10.81	Carvacrol	1302	0.60
10.97	Varamol	1309	2.95
11.37	Eugenol	1356	3.96
11.48	Capric acid	1364	12.21
11.53	Methyl eugenol	1403	0.37
12.16	(E)-Geranylacetone	1445	0.20
12.20	(E)-Isoeugenol	1451	0.47
13.07	Lauric acid	1565	5.77
13.64	Apiol	1677	0.46
14.54	Myristic acid	1775	1.29
15.69	Palmitic acid, methyl ester	1921	t
15.85	Isophytol	1948	t
15.93	Palmitic acid	1959	3.49
16.92	Phytol	2122	1.21
17.04	Linoleic acid	2132	0.66
17.08	Oleic acid	2140	0.76
17.18	Stearic acid	2159	t

Total composition of oil = 92.90%; t = trace <0.2%

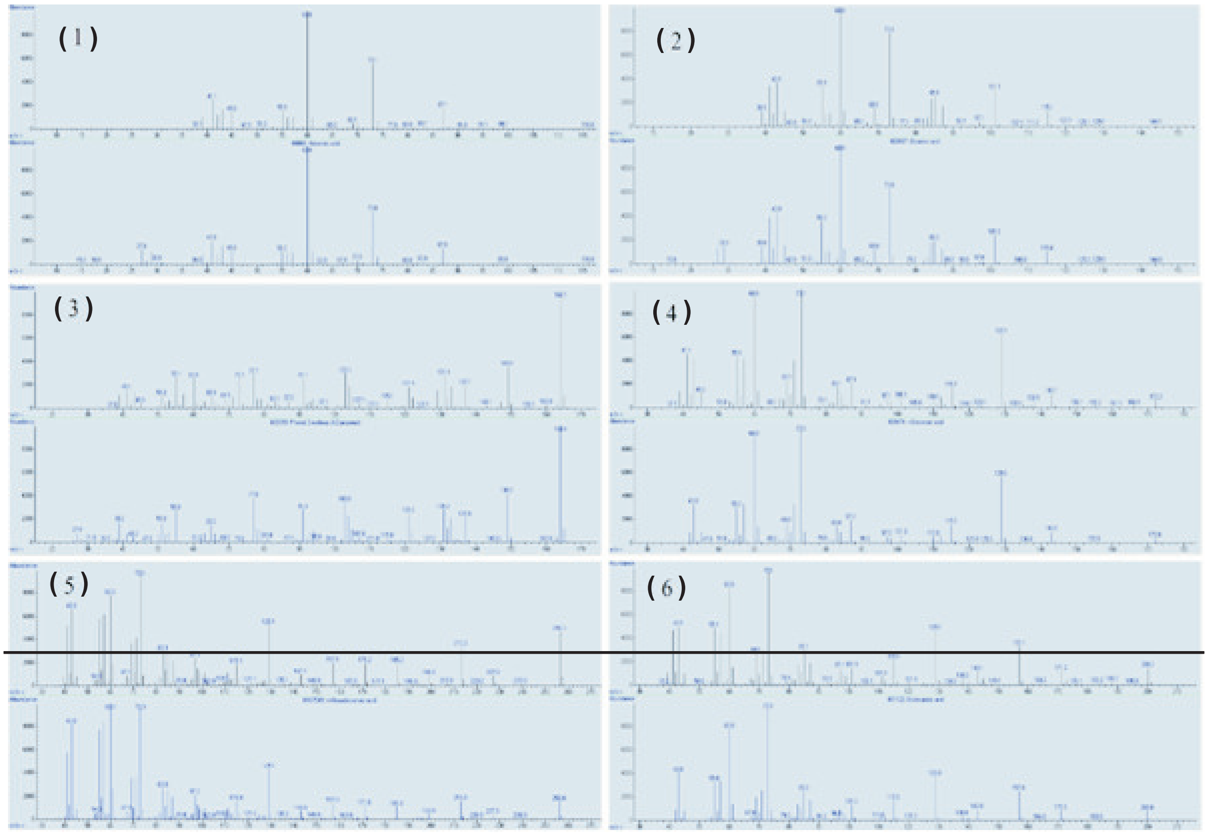

Fig. 1. Matching mass spectra of 6 major compounds with NIST-11 standards. **(1)** = caproic acid, **(2)** = caprylic acid, **(3)** = eugenol, **(4)** = capric acid, **(5)** = palmitic acid; **(6)** = lauric acid.

Table 2 demonstrates the percentages of different classes of volatile oil constituents. The most dominating classes were fatty acids (71.91%), oxygenated monoterpenes (7.87%), aldehydes (3.97%) and phenols (3.55%). Obviously, the most dominant class of fatty acids is one of the key factors responsible for the antibacterial activity of this oil as lauric, palmitic, palmitoleic, linoleic and oleic acids have been extensively reported as antimicrobial agents (Anneken *et al*., 2006; Dilika *et al*., 2000; Ouattara *et al*., 1997). Furthermore, a high percentage of fatty acids especially palmitic acid serves as a precursor to produce the required prostaglandins for inflammatory activity (Aparna *et al*., 2012). Several compounds present in this oil are biologically important and are extensively used for a wide range of applications. However, the presence of eugenol is noteworthy because

Table 2. Classification of volatile constituents

Class of volatile constituents	Serial number of the constituents	Percentage (%)
Oxygenated monoterpenes	14, 19, 20, 22, 24, 26, 28, 29, 30, 32	7.87
Oxygenated sesquiterpenes	35, 37	1.21
Fatty acids	6, 11, 15, 17, 21, 27, 31, 33, 36, 38, 39, 40	71.91
Fatty acid, methyl esters	7, 34	0.22
Phenols	12, 25	3.31
Aldehydes	3, 5, 8, 18	3.43
Hydrocarbons	13, 23	1.66
Others	1, 2, 4, 9, 10, 16	3.29
Total		**92.90**

of its well-known antimicrobial and anthelmintic activities but it may also cause hepatoxicity (Janes *et al*., 2005), while the pharmaceutical administration requires strict and safe dosage experiments prior to commercialization.

Antibacterial activity. Previous literature revealed its steam distilled oil in petroleum ether exhibited activity against *V. cholera* and *M. pyrogenes* var aureus whereas its fruit oil showed activity against *V. cholera* and *S. aureus* (Khan *et al*., 1993; Gaind and Budhiraja, 1967). In another study, various extracts of its fruit were evaluated against various bacterial strains (*S. flexneri, S. aureus, S. typhi, P. aeruginosa, K. pneumoniae, P. vulgaris, E. aerogenes*) where ethyl acetate extract showed maximum activity against *E. aerogenes* (28 mm) at the concentration of 150 mg and methanol extract showed maximum activity against *K. pneumoniae* (21 mm) at 250 mg (Sudhanshu *et al*., 2012). Another study on its methanolic fruit extract showed the highest inhibitory activity against *Bacillus subtilis* (12 mm) among other tested strains *(Klebsiella pneumoniae*, *Micrococcus luteus*, *Salmonella paratyphi*, *Staphylococcus aureus* and *Escherichia coli*) (Peerzade *et al*., 2018).

In our study, the fruit oil was tested against various drug-resistant strains and the most promising activity was observed against *P. aeruginosa* (21 mm), weak activity was found against *E. faecalis* (11 mm) and *MRSA* (10 mm) whereas no activity was found against *E. coli* and *K. pneumoniae* at the maximum dose level of 0.48 mg (100 µL) as shown in Table 3. The activity was observed in dose dependent manner in almost all cases where Fig. 2 shows the activity of oil against susceptible strains.

Table 3. Antibacterial activity of oil against various strains

Bacterial strain	Volume and amount of dissolved oil				Positive control (Amoxicillin)	Negative control (*n*-hexane)
	25µL (0.12 mg)	50µL (0.24 mg)	75µL (0.36 mg)	100µL (0.48 mg)	(100 µg)	
	Zone of inhibition (mm)					
P. aeruginosa	13	16	19	21	8	0
K. pneumoniae	0	0	0	0	0	0
E. coli	0	0	0	0	0	0
E. faecalis	0	0	9	11	12	0
Methicillin-resistant S. aureus	0	7	7	10	14	0

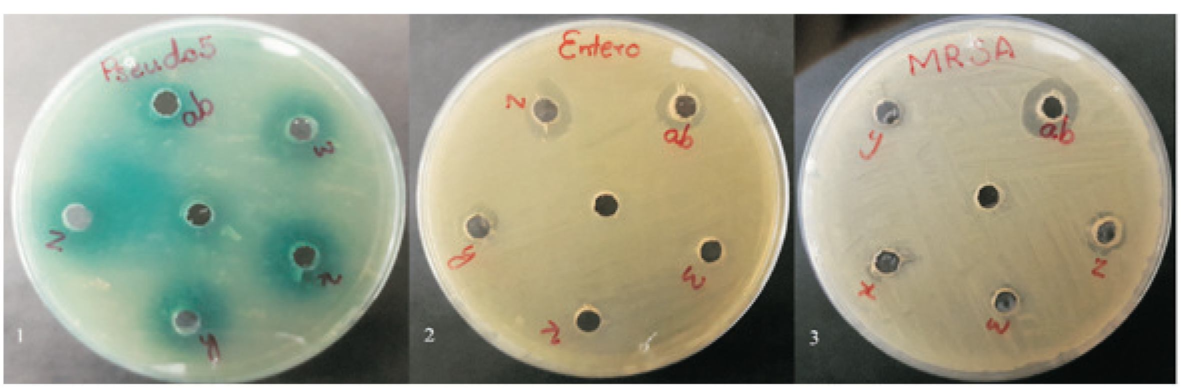

Fig. 2. Antibacterial activity of oil **(1)** = *P. aeruginosa*; **(2)** = *E. faecalis*; **(3)** = *Methicillin-resistant*, *S. aureus*; ab = antibiotic; z = maximum dose of sample = (0.48 mg).

Fruit oil of *W. coagulans* plant has shown a significant potential against *P. aeruginosa*. However, the reason behind the difference in activity among various other strains may be due to the fact that volatile oils act differently on different microbes. Obviously, numerous important antimicrobial fatty acids, lauric, palmitic, palmitoleic, linoleic and oleic acids etc. (Anneken *et al*., 2006; Dilika *et al*., 2000; Ouattara *et al*., 1997) and other bioactive constituents such as thymol and carvacrol, that generally act as membrane permeabilizers (Lambert *et al*., 2001) are responsible for the activity and their possible synergistic action could have imparted their role in overall antimicrobial activity of this oil.

Conclusion

In the current and detailed study on the volatile oil composition of *W. coagulans* (fruits), 40 constituents were identified and most dominating were caprylic acid (28.58%), caproic acid (16.88%), capric acid (12.21%), lauric acid (5.77%), palmitic acid (3.49%), eugenol (3.96%) and varamol (2.95%). Fatty acids (71.91%) and oxygenated monoterpenes (7.87%) were found to be the most dominating classes. Antibacterial activity demonstrated its significant potential whereas the strongest activity was observed against *P. aeruginosa* (21 mm) that shows the potential of this oil especially against this strain.

Conflict of Interest. The authors declare that they have no conflict of interest.

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