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Research Article

Essential Oil Composition and Antibacterial Activity of *Alstonia scholaris* (flower) from Pakistan

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ABSTRACT
This study investigated the essential oil composition and antibacterial activity of the flowers of *Alstonia scholaris*. Microwave-assisted extraction of essential oil was performed, and the extraction process resulted in a 0.218% (w/w) yield. Gas chromatography mass spectrometry (GC-MS) revealed twenty-seven constituents, and confirmation was performed by calculating their retention indices. The most abundant compounds were: 2-Hexenal, (E)- (12.455%), 3-Hexen-l-ol, (Z)- (11.347%), Heneicosane (6.471%), Hexanal (5.913), Tricosane (5.879%), and 2-Methoxy-4-vinylphenol (4.557%). The agar disc diffusion assay was used to determine the antibacterial activity of the volatile oil against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa, and* zones of inhibition were observed against these strains as 11mm, 11mm, 10mm, and 9mm, respectively. The presence of various well-known antibacterial agents shows an obvious relationship with the activity of the essential oil.

Keywords: Essential oil, *Alstonia scholaris*, Apocynaceae, microwave-assisted extraction, antibacterial activity

INTRODUCTION
Alstonia scholaris belongs to the Apocynaceae family (Endress et al., 2014). The scientific name of this plant honors Professor Alston, while the species name *scholaris* was given due to its traditional use in making blackboards in Asia (Arulmozhi, 2007). The plant has been employed for the remedy of cancer, jaundice, and malaria, and other common names for this tree are Indian Devil tree or Blackboard tree (Burkill, 1996). The plant generally grows in mountainous regions of the Asia Pacific, China, and India, and lowlands. Mainly humid regions such as West Bengal and the West Coast forests of southern India are key areas for the growth of this plant (Gardner, 2011). Flowering season starts in October, and greenish white flowers produce a strong aromaticity. Cymose type inflorescence is observed. It is a globular tree that rises to 40 m in height. Rainforests and terrestrial areas with monsoon-type climate are the native habitat (Khyade et al., 2014). It possesses cytotoxic, anti-inflammatory, antimicrobial, antidiarrheal, analgesic, and anticancer properties (Gandhi and Vinayak, 1990; Bhandary, 2020; Shang, 2010; Linn, 2010; Bagheri, 2016). Essential oils are extracted through numerous techniques, including expression, hydrodistillation, supercritical fluid extraction, steam distillation, Soxhlet extraction, ultrasonic extraction, and microwave-assisted extraction (MAE) (Reyes-Jurado, 2015). MAE has various benefits over other extraction methods; also, it is widely employed in research due to its accuracy and better yield. It is a green extraction technique that requires less solvent and minimum time, usually between 30-60 minutes, as compared to 5-6 hours required

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by steam and hydrodistillation (Sattar, 2024). Moreover, MAE requires less solvent, the quantity of waste is minimal, no harmful chemicals are used, and zero to none toxic solvents are involved, thus making it safe for both the environment and the chemist (Bagade and Patil, 2021).

Essential oils possess numerous bioactive properties, especially antimicrobial activity against various disease-causing and foodborne pathogens (Intisar, 2012; Aziz, 2021). Previously, volatile oils from various parts of *Alstonia scholaris* have been studied (Dung, 2001; Singh, 2020; D'Cruz and Ambaktar, 2016; Hussain et al., 2013; Ali et al., 2020). Most of the studies on essential oil extraction from flowers were based on traditional hydro- and steam distillation methods (Dung, 2001; Singh, 2020; D'Cruz and Ambaktar, 2016). However, hardly any study on microwave-assisted essential oil extraction of flowers has been reported for *Alstonia scholaris*. Further, there will hardly be a report on the flower essential oil composition of this plant of Pakistani origin. Hence, this study was specifically conducted to fill the gap in knowledge and improve the extraction technique in terms of extraction time and yield. Additionally, the difference in chemical composition was compared along with the antibacterial activity.

MATERIALS AND METHODS

Essential Oil Extraction

Fresh flowers of this plant were collected from the plants grown in the School of Chemistry, University of the Punjab, Lahore. A fresh sample weighing 100 grams was taken and finely chopped. Distillation was carried out using a modified household microwave (Orient, Model OM46SS, Pakistan) for around 40 minutes. To prevent overheating, the temperature was carefully controlled. The essential oil was collected using dichloromethane, and for GC-MS analysis, the sample was kept refrigerated at –10℃. Moreover, the solvent was evaporated at low temperature (below 40℃) for performing the antibacterial assay.

GC-MS Analysis

An Agilent (5677 A series) equipped with a DB-5MS capillary column was used. The helium flow rate was set at 1 mL/min, and a temperature of 40℃ was used to start, which was held for 4 minutes, then raised at 10℃/min to 280℃, where it was held for an additional 2 minutes. MSD ChemStation with NIST library 2011 was used to identify the compounds present in the essential oil. Alkane series obtained from Sigma Aldrich (C_7-C_{30}) were run under identical conditions and used for calculating retention indices, which were then matched with the reference values present in the literature.

Antibacterial Activity

Strains, namely, *Escherichia coli, Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and *Staphylococcus aureus*, were obtained. The sample was prepared using dimethyl sulfoxide (DMSO) as the solvent, which was also used as the negative control. An agar disc diffusion assay was employed. Luria agar was used, and it was poured into sterilized petri dishes. Microbes were inoculated in these petri dishes. Then filter paper disc was employed, and 20 µL of dissolved oil was introduced onto it and placed onto the agar. Following incubation at 37℃ for 24 hours, the zones of inhibition were recorded.

RESULTS AND DISCUSSION

The essential oil with a pale yellow color was obtained from the plant sample. The quick microwave-assisted extraction (MAE) procedure required only 60 minutes for the extraction. Moreover, the extraction yield in this study was 0.218%. (w/w), which is higher than earlier reports, which ranged between 0.01% and 0.03% (w/w) in the case of fresh flowers (Dung, 2001; Singh, 2020). In total, 27 compounds were identified, with the most abundant being 2-Hexenal, (E)- (12.455%), 3-Hexen-l-ol, (Z)- (11.347%), Heneicosane (6.471%), Hexanal (5.913), Tricosane (5.879%), and 2-Methoxy-4-vinylphenol (4.557%). Table 1.

FEO of *A. scholaris* obtained via hydrodistillation contained trans-linalool oxide (furanoid) (7.0-7.5 %), cis-linalool oxide (furanoid) (7.3-12.2 %), and linalool (13.6-14.3%), along with terpinen-4-ol (7.9-10.6 %), and α-terpineol (6.3-6.6 %), etc. (Singh, 2020). Upon hydrodistillation of petal and corolla tubes, the main compounds were: linalool (35.7%), cis- and trans-linalool oxides (14.7%), and α-terpineol (12.3%), etc. (Dung, 2001). However, in another study, steam-distillation of flowers showed eucalyptol (1,8-cineole) and linalool as major constituents (D'Cruz, 2016). Compared to previous studies, this study identified a total of 27 constituents, among which common compounds included Furfural, eucalyptol, and cis- and trans-linalool oxides, terpinen-4-ol, α-terpineol, etc.

The relative mass spectra of six major compounds from the essential oil of *A. scholaris* have been provided as under;

2-Hexenal, (E)-: RT 4.117 min: m/z 98.10 (M^+, 28), 41.10 (100), 49.10 (11), 55.10 (99), 63 (3), 69.10 (92), 83 (77), 83.10 (80).
3-Hexen-l-ol, (Z)-: RT 4.311 min: m/z 100.0 (M^+, 3), 31.10 (14), 38.10 (3), 41.10 (89), 44.10 (6), 55 (50), 58 (2), 63 (2), 67 (100), 70 (15), 79 (3), 82 (100).
Heneicosane: RT 21.474 min: m/z 296.40 (M^+, 4), 43 (67), 57 (100), 71 (80), 85 (54), 127 (8), 141 (8), 155 (5), 169 (4), 197 (2), 211 (2), 239.30 (2), 253.30 (2).
Hexanal: RT 2.760 min: m/z 100 (M^+, 1), 38 (3), 41 (83), 44 (100), 53 (5), 56 (99), 67 (17), 72 (30), 79 (1).
Tricosane: RT 23.233 min: m/z 324 (M^+, 1), 43 (58), 57 (100), 71 (75), 85 (60), 99 (19), 113 (11), 127 (8), 141 (6), 155 (4), 169 (3), 183 (3), 197 (2), 211 (2), 225 (2), 239 (2), 253 (2), 267 (1), 281 (1).
2-Methoxy-4-vinylphenol: RT 13.023 min: m/z 150 (M^+, 100), 39 (10), 51 (12), 63 (8), 67 (7), 73 (5), 77 (38), 107 (36), 135 (82).

Table 1. Chemical composition of the essential oil of Alstonia scholaris

Sr. No.	Retention time (min)	Names of compounds	Calculated Retention indices (RI_{cal})	Retention indices from literature (RI_{Lit})	Relative abundance (%)
1.	2.76	Hexanal	805	805	5.91
2.	3.78	Furfural	844	844	3.08
3.	4.06	3-Furaldehyde	855	846	0.24
4.	4.11	2-Hexenal, (E)-	857	857	12.45
5.	4.31	3-Hexen-l-ol, (Z)-	864	864	11.34
6.	5.97	α-Pinene	932	932	0.87
7.	8.14	Eucalyptol	1031	1031	3.56
8.	8.94	cis-Linalool oxide	1074	1068	2.29
9.	9.23	trans-Linalool oxide	1090	1089	2.07
10.	9.55	β-linalool	1108	1108	2.66
11.	10.69	Epoxylinalol	1179	1183	0.57
12.	10.82	Terpinen-4-ol	1187	1187	2.93
13.	11.12	α-Terpineol	1205	1205	1.80
14.	13.02	2-Methoxy-4-vinylphenol	1338	1336	4.57
15.	13.54	α-copaene	1376	1376	0.87
16.	14.53	trans-Geranylacetone	1453	1453	0.56
17.	14.92	(+)-epi Bicyclosesquiphellandrene	1484	1481	2.53
18.	15.09	cis-α-Bisabolene	1498	1498	0.49
19.	15.19	α-Farnesene	1506	1506	0.16
20.	19.60	2-Heptadecanone	1904	1902	2.63
21.	19.82	Hexadecanoic acid, methyl ester	1927	1927	0.43
22.	21.42	9,12-Octadecadienoic acid, methyl ester	2093	2093	1.05
23.	21.47	Heneicosane	2099	2100	6.47
24.	22.99	9-Tricosene (Z)-	2271	2271	0.26
25.	23.23	Tricosane	2299	2300	5.87
26.	24.65	Behenic alcohol	2473	2473	0.74
27.	27.15	Supraene	2808	2808	1.15

This study also aided in identifying the biologically active and potentially therapeutic compounds such as 2-Hexenal, (E)-, 3-Hexen-l-ol, (Z)-, Heneicosane, Hexanal, and 2-Methoxy-4-vinylphenol. 2-Hexenal, (E)- is used as an antifungal agent to preserve fruits and vegetables, such as its vapor treatment on tomato seedlings prevents tomatoes from stress and fungal attack. They also act as defensive or attractive secretions in insects; therefore can be used for their controlled development, such as in male and female bed bugs. 2-Hexenal is responsible for the lure (Onnink et al., 2017). The second abundant compound, 3-Hexen-l-ol, (Z)- is used in agriculture and forestry, as increasing its release from plants can enhance plants' defense, as it reduces the number of herbivores that attack up to 90% (Arey, 1991). Due to its aroma, it is used as a flavoring agent and in perfumes and deodorants (Bedoukian, 1971). Heneicosane showed strong antimicrobial activity against Aspergillus fumigatus 29 ± 0.86 mm and *Streptococcus pneumoniae* 31 ± 0.64 mm, respectively, at the concentrations of 10 µL/mL(Vanitha, 2020). Hexanal showed activity against the bacterial strains that are the agents of respiratory tract and human intestinal infections (Nakamura and Hatanaka, 2002). 2-Methoxy-4-vinylphenol is an organic compound that is used in the treatment of pancreatic cancer due to its anti-inflammatory activities and causes cessation of the cell cycle (Kim, 2019).

Table 2 provides the classification of the essential oil components. Aldehydes, such as 2-Hexenal, (E)-, hexanal, furfural, and 3-furaldehyde collectively constitute 21.69% of the flower oil. Other groups are alcohols and phenols (20.79%), monoterpenoids (14.83%), alkanes (12.35%), ketones (5.24%), Sesquiterpenoids (4.23%), and monoterpenes (0.87%). Aldehydes are tested as antitoxins as they have been reported to work against toxins produced by microbes. They are tested positive for their antigerminative properties (Bowles and Miller, 1993). It was reported that hexanal showed bacteriostatic action at a concentration of less than 12.5 µg mL^{-1} (Nakamura and Hatanaka, 2002). Monoterpenoids are reported to have widespread applications in medicine (Volcho, 2018). Linalool was tested against 63 bacterial strains, and it showed antibacterial activity with a broad spectrum, with a zone of inhibition ranging from 7-11 mm (Kotan et al., 2007). Terpinen-4-ol is a monoterpenoid alcohol that has shown antibacterial activity against *S. agalactiae* (Zhang, 2018). α-Copaene is a sesquiterpenoid; it was tested against four bacterial strains, and the mechanism of its antibacterial activity was studied. It was recorded that a 4 µL/mL concentration of α-Copaene inhibited the bacterial growth in beef soup (Chen, 2024). α-Pinene is a monoterpene that has been reported to have MBC values of 210 µg/mL for MRSA and 100 µg/mL for *Streptococcus pyogenes* (Yang et al., 2015).

Table 2. Classification of essential oil constituents

Sr. No	Class of Volatile Constituents	Percentage (%)
1	Monoterpenoids	15.71%
2	Sesquiterpenoids	4.24%
3	Alkanes	12.35%
4	Aldehydes	21.69%
5	Ketones	5.25%
6	Alcohol and phenols	20.66%

Antibacterial Activity

This study showed the positive results of antibacterial activity against all four strains. The maximum zone of inhibition was shown by *E. coli* and *K. pneumoniae,* which is 11 mm, and the least was given by *P. aeruginosa,* which is 9mm. Antibacterial activity is shown in Table 3.

Table 3. Antibacterial activity of the essential oil of *Alstonia scholaris*

Sr. No.	Bacterial strains	Zones of inhibition (mm) at 20 µL dissolved oil		
		1st	2nd	3rd
1	*Escherichia coli*	11	11	10
2	*Klebsiella pneumoniae*	12	11	11
3	*Staphylococcus aureus*	10	10	10
4	*Pseudomonas aeruginosa*	09	08	09

Ganjewala et. al. tested the antibacterial activity of leaves, follicles, and latex of *Alstonia scholaris* against four strains Gram *+ve (B. subtilis and S. aureus) and Gram-negative (E. coli, P. aeruginosa) bacteria.* Methanol extract of these components was used to find the antibacterial activity of these parts by the well diffusion technique. The methanolic extract of latex (100mg/mL) showed the greatest zone of inhibition, 21mm, for *B. subtilis* (a gram-positive bacterium). The minimum zone of inhibition was measured against *P. aeruginosa* (a gram-negative bacterium), which is 8mm (Ganjewala and Gupta, 2013). Another study gives the results of antibacterial activities of roots, stem bark & leaves of various solvent extracts (Water, Hexane, Methanol, Benzene, Isopropanol, Ethyl acetate) of *Alstonia scholaris* against Gram-positive (*Lactococcus lactis*, *Staphylococcus aureus,* and *Bacillus cereus*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Aeromonas sp.*, *Escherichia coli*, *Enterobacter aerogenes,* and *Proteus mirabilis*) by the well diffusion method. Among root extracts, 11.51 mg/100µL of methanol extract showed the maximum zone of inhibition (18mm) against *E. coli*. Also, the 31.82mg/100µL of methanolic extract of leaf showed maximum zone of inhibition (13.5 mm), but against *Lactococcus lactis*. The extract of stem bark in isopropanol 20.28mg/100µL showed maximum zone of inhibition, and it is also against *Lactococcus lactis* (13.3 mm) 9Misra, 2011). Different extracts of *Alstonia scholaris* fruit were tested for their antibacterial activity against *Staphylococcus aureus, Bacillus cereus, Lactococcus lactis, Pseudomonas aeruginosa, Escherichia coli,* and *Salmonella typhi* by agar well diffusion method. 54mg/100µL of butanol extract and 40mg/100µL of benzene showed maximum antibacterial activity against *Staphylococcus aureus and Lactococcus lactis,* with a maximum zone of inhibition of 25mm, respectively. Butanol extract also showed antibacterial activity against *Salmonella typhi* with a zone of inhibition of 23mm (James et al., 2012).

CONCLUSIONS

A total of 27 volatile compounds were identified from the essential oil of the flower of *Alstonia scholaris*. The predominant classes were aldehydes, alcohols, phenols, and monoterpenoids. Many of the compounds were aromatic and biologically active. The EO shows excellent activity against all strains (*E. coli, Klebsiella pneumoniae, Staphylococcus aureus, and Pseudomonas aeruginosa*) with the agar disc diffusion method. The antibacterial activity of compounds against different strains suggests the high potential of this plant.

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AUTHOR CONTRIBUTIONS

Muhammad Saleh Zaman & Azeem Intisar: Conceptualization, Literature survey, writing original draft, experimental work, Data interpretation; Farhana Ahmad & Hamna: Data interpretation, Visualization, Validation, writing reviewing & editing; Zeeshan Muthair, Ejaz Ahmed: Visualization, Validation, writing reviewing & editing; Ahsan Sharif & Shahbaz Ahmad: Project administration & correspondence, visualization, validation, writing reviewing and editing.

COMPETING INTEREST

No conflicts of interest have been disclosed by the authors.

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