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GC-MS Profiling of Anti-bacterial Metabolic Compounds from the Extract of *Azadirachta indica*

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Abstract

Azadirachta indica is a very common plant used very frequently due to its medicinal significance. The antibacterial activities of 0.001, 0.01, 0.1, 1.0 and 10 mg/mL of the plant extract were determined against different pathogenic bacteria. Concentration of 0.01 mg/mL killed the *E. coli, E. aerogenes, P. stuartii* and 10, 1.0 and 0.1 mg/mL were very effective against the E. cloacae, K. pneumoniae and P. mirabilis and killed them 100% in culture plates. The plant extracts were analyzed for the characterization of the different antimicrobial compounds through gas chromatography-mass spectrometry (GC-MS). An array of antibacterial compounds such as azulene, tetrasiloxane, phthalic acid, cyclopentasiloxane, hexadecanoic acid, spiropentane, dioctyl phthalate were detected in the plant extract through GC-MS. The antibacterial activities of the plant extracts were might be because of their compound which had been reported previously as well as antimicrobial compounds.

Keywords: Azadirachta indica, antibacterial activities, Enterobacteriaceae, GC-MS, secondary metabolites, antibiotic resistant genes

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1. INTRODUCTION

The seeds, fruit, oil, leaves, bark and roots for their useful medicinal properties[1]. Extract of *A. indica* showed strong activity against so many despise. Researches on neem have been intensified in the last 20 years, researchers discovered numerous agricultural and medical properties. Neem shows effects against ulcers and different types of gastric disorders help improve the digestive system, assisting and removing toxins and pathogenic bacteria. In addition, neem used to remove parasites; it kills both internal and external parasites [1]. Extracts have hormone mimics that can inhibit the ability to feed and prevent the eggs from hatching [2]. During the last several years, studies show that neem components have anticancer activities; it can inhibit breeding and invasion of cancer cells [3]. Plants produce these secondary metabolites for their protection against biotech and abiotic stressors [2]. However, the metabolic compounds can also be used against a variety of human disorders including infectious diseases. In old ages, the plant extracts were obtained through simple distillation but now as the scientific equipment has been developed; more refined products can be extracted [4]. The *A. indica* has lots of phytochemical used for medicinal purposes for centuries. Phytochemicals are chemicals extracted from the plants are classified as primary and/or secondary metabolites [5]. However, these are mostly secondary metabolites used for variety of purposes

including medicine [6]. Plants have been always used against bacteria and bacterial infectious diseases because plants have a greater range of secondary metabolites including terpenoids, quinones, tannins, alkaloids, steroids, glycosides [7].

Enterobacteriaceae is a common family of Gram-negative facultative anaerobic, rod-shaped, non-spore-forming bacteria. The members of this family are including *Biostraticola*, *Arsenophonus, Klebsiella, Moellerella* and many others [8]. The *Enterobacteriaceae* members are catalase positive, oxidase negative, all other except *plesiomonas* follow the Embden–Meyerhof pathway for sugar metabolism and acid production from glucose fermentation. *Enterobacteriaceae* is among the larger bacterial family consisting of 51 genera [7].

Increased resistance to antibiotics and the shortage of new antimicrobials have been recognized for a long time [7,8]. The bacterial infection can be minimized through the development of affordable and economic alternative medicines especially in poor countries, where deaths account from infections [2]. *Enterobacteriaceae* has been among the larger family having 51 genera. The common infections caused by a different member of *Enterobacteriaceae* are including skin and other soft-tissue infections, intra-abdominal infections, endocarditis, infections in the lower respiratory tract, urinary tract infections and ophthalmic infection [8]. Some isolates of *Enterobacteriaceae*, cause skin infections, are becoming resistant against a variety of antimicrobial compounds including gentamicin, penicillin, amikacin, tobramycin, ciprofloxacin and some other antibiotics. The extensive usage of the antibiotic and their contaminations in the environment make the bacteria resistant against the conventional antibiotic agents [8]. The problem of antibiotic resistance in humans and animals will persist for a long time [9]. Based on the issue of antibiotic-resistant bacteria, there is a need to generate another alternative drug to treat infectious bacterial diseases [10]. The studies showed that plant extracts are highly efficient against infectious bacterial diseases [11]. Saudi Arabia has characterized a wide variety of plants containing a lot of bioactive metabolites [12]. However, these plant extracts are still largely be used as a traditional medicine to cure bacterial infectious diseases. The study has been designed to evaluate the antibacterial effects of neem extract against infectious diseases caused by *Enterobacteriaceae* family.

2. MATERIALS AND METHODS

2.1 Samples collection

Azadirachta indica plants were collected from a different region of Jeddah, KSA, brought to the laboratory of the Department of Biological Sciences, King Abdulaziz University Saudi Arabia. The plants were washed with deionized distilled water to remove the dust, debris and soil particles from the surface of the plants. The sample plants were dried in by spreading the table leaving for 24 h. Further *A. indica* plant was shed dried by keeping the plants in the shed under pressure and left to dry, then crushed, ground by mortar and pestle, and extracted using Hexane, then the second extract with methanol.

2.2 Extraction of compounds from A. indica

The plant sample was wrapped in filter paper and pressed under pressure to absorb the moisture. The sample was placed in the shade until it was dried completely to obtain a homogenous sample and to enhance the kinetics of analytic extraction. The dry plant sample was crushed using mortar and pestle into a fine powder. Proper actions were taken to assure bioactive constituents were not distorted, lost and/or destroyed while drying and extraction of plant metabolites. Different solvent systems are available to extract the bioactive compound from natural products. Polar solvents such as different percent of n-hexane and methanol to extract the hydrophilic compounds. The metabolites from the plants were extracted using the solvent-solvent extract method. The extraction was carried out by 70% MeOH.

2.3 GC-MS analysis of compounds from A. indica

The *A. indica* plants were dried, ground and was extracted in 70% MeOH. The extract was dried and was suspended in 100% MeOH and filtered through 0.45 µm filter. The helium gas (He) was used as career gas at 1 mL/min flow rate and 2 µL sample volume was injected at the split ratio of 10:1. The column was temperature was set for 2 min at 60°C and then increased up to 160°C at a rate of 5°C /min for 5 min. In the

electron ionization system, the ionization energy to detect the ions was 70 eV in electron impact mode. The spectrum of detected compounds in extract were compared with library present in NIST library.

2.4 Antibacterial activities of extract

Antibacterial assessment for the extract was performed by growing all test bacterial strains in LB broth to get an OD600 nm of 0.8~1.0. an aliquot of 10 µL of each culture bacteria was then spread on Muller Hinton agar plate. Wells were cut off from the agar surface (6 mm diameter) and 30 µL of test samples added in each well. The cultures were incubated for 48 ± 2 h at 37°C and zones of inhibition were measured. Furthermore, minimal inhibitory concentration (MIC) of the compound was determined by adding different concentrations of a compound in 5 mL LB media. The respective bacterial species were inoculated and inoculated on a shaker (200 ± 20 rpm) at 37°C and the growth determined at an optical density (OD) 600 nm using a spectrophotometer. The MIC was the concentration at which no visible growth was recorded at respective OD.

3. RESULTS AND DISCUSSIONS

3.1 Identification and characterization of compound through GC-MS

The composition of metabolites of the neem plant was determined through GC-MS profiling. The GC-MS analysis of the plant extract revealed 14 different bioactive compounds the main metabolites were including undecane, azulene, decane, tetrasiloxane, hexamethyl-3,5-bis(trimethylsiloxy), phthalic acid, Cyclopentasiloxane, hexadecanoic acid, spiropentane, dioctyl phthalate. The GC-MS chromatogram of the methanol extract of neem plants showed 14 peaks which indicated the presence of fourteen metabolites compounds. The spectra of the compounds were matched with the National Institute of Standards and Technology libraries. The compounds detected are presented in Table 1. The component metabolites that were present in the methanolic extract were identified according to their retention time. The major biologically active compounds were including undecane, azulene, tetrasiloxane, phthalic acid, cyclopentasiloxane, hexadecanoic acid, spiropentane, dioctyl phthalate. These compounds were biologically active as antifungal, anti-influenza, antimicrobial, and antioxidants.

In the compounds obtained, some components were biological active; anti-inflammatory, antifungal, antioxidant and anticancer. All these compounds are important in the formulation of different medicines. Hexadecanoic acid, methyl ester is used as an antioxidant, anti-inflammatory, possess hypolipidemic properties and is also used as an antimicrobial agent[13]. The cyclopentasiloxane has antioxidant activity, is anticarcinogenic; used as dermatitigenic flavor and exists in human blood and urine where it serves as endogenous peroxisome proliferator-activated receptor ligand[14]. The Phthalic acid has been reported as strong antimicrobial agent against different pathogenic bacteria. In addition, its antioxidant properties and anti-cancerous activities has also been reported[15].

3.2 Antibacterial activity of plants extract

A. indica was investigated to evaluate the antibacterial activity against the different bacterial of *Enterobacteriaceae* family including *E. cloacae, E. coli, E. aerogenes, P. stuartii, K. pneumoniae* and *P. mirabilis* using disc diffusion method. Different concentrations of the neem plant extracts such as 0.001, 0.01, 0.1, 1.0 and 10 mg/mL were evaluated and the results (Table 2) revealed that all dilution of the extract suppressed the pathogenic bacterial species i.e., *E. cloacae, E. coli E. aerogenes, P. stuartii, K. pneumoniae,* and *P. mirabilis* (Fig. 1). The dilutions of plant extract from 10 to 0.1 mg/mL were more effective against all the tested pathogenic bacteria. The dilution 0.01 mg/mL effectively killed the *E. coli E. aerogenes, P. stuartii* and the dilution 0.001 mg/mL was effective against the *E. aerogenes* and *P. stuartii.* Although the dilutions 10, 1.0 and 0.1 mg/mL were very effective against the *E. cloacae, K. pneumoniae* and *P. mirabilis* and killed them 100% in culture plates. However, the dilution 0.01 mg/mL and 0.001 mg/mL were not very significant against *E. cloacae, K. pneumoniae* and *P. mirabilis.*

Table 1. The GC-MS profile of the metabolites of MeOH extract of *A. indica*

Peak#	Ret.Time	Area	Height	A/H	Name
1	6.234	211818	48710	4.35	Undecane, 3,8-dimethyl-
2	7.589	332727	99237	3.35	Azulene
3	10.592	30663	18883	1.62	Hexane, 3,3-dimethyl-
4	11.899	88423	45620	1.94	Tetrasiloxane, 3,5-diethoxy-1,1,1,7,7,7-hexamethyl-3,5-bis(trimethylsiloxy)-
5	13.997	75870	44953	1.69	N-(Trifluoroacetyl)-N, O, O, O-tetrakis(trimethylsilyl)norepinephrine
6	15.814	59685	36315	1.64	Cyclohexasiloxane, dodecamethyl-
7	16.855	462396	232197	1.99	Hexadecanoic acid, methyl ester
8	17.293	50765	28423	1.79	Phthalic acid, cyclobutyl isobutyl ester
9	17.436	38359	22206	1.73	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-
10	18.575	31882	17013	1.87	Spiropentane, propyl-
11	18.639	719885	305084	2.36	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-
12	18.758	765591	397830	1.92	Phytol
13	18.862	169775	86090	1.97	Hexadecanoic acid, 15-methyl-, methyl ester
14	18.925	30501	18011	1.69	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-

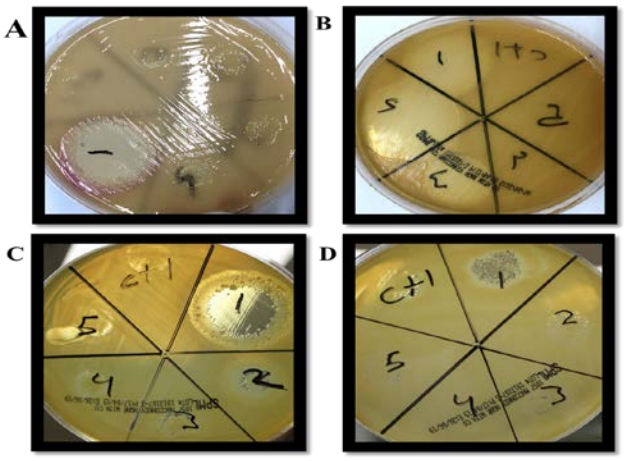

Fig. 1. explain each figure separately

A. indica has been tested to evaluate antibacterial activity against several bacteria of the *Enterobacteriaceae* family such as *E. cloacae, E. coli, E. aerogenes, P. stuartii, K. pneumoniae* and *P. mirabilis* using the disc diffusion method. The results were effective, and extract killed 100% pathogenic bacteria [16]. The bacterial strains included in this study were selected for their importance in infectious diseases. These results are consistent with the results of [17]. The antibacterial activities of the extract of the *P. granatum* showed some variation which could be due to a significant difference in the types of bacterial strains, components and extraction method applied for analysis [18]. In addition, variability in the MIC of different plant extracts can derive from the difference in their chemical components and from the volatile nature of their components[19].

Table 2. Antibacterial activities of the extract of *A. indica* against different member of *Enterobacteriaceae* family

Strains	1	2	3	4	5	Control
E. cloacae	+ve	+ve	+ve	-ve	-ve	-ve
E. coli	-ve	+ve	+ve	+ve	-ve	-ve
E. aerogenes	+ve	+ve	+ve	+ve	+ve	-ve
P. stuartii	+ve	+ve	+ve	+ve	+ve	-ve
K. pneumoniae	+ve	+ve	+ve	-ve	-ve	-ve
P. mirabilis	+ve	+ve	+ve	-ve	-ve	-ve

The GC-MS analysis of the extract showed that *A. indica* produces a versatile array of secondary metabolites azulene, tetrasiloxane, phthalic acid, Cyclopentasiloxane, hexadecanoic acid, spiropentane, dioctyl phthalate. These metabolites are reported to have antibiotic characteristic against wider range of bacteria [20].the CG-MS profile showed the presence of the phthalic acid and Cyclopentasiloxane compounds in the extract have strong antibacterial activities against a wide range of gram-positive and gram-negative bacteria [19].in addition, previous study reported the phthalic acid extracted from plant showed very strong antibacterial activity in a disc diffusion method activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella flexneri*, and *Klebsiella pneumonia* [21]. The wide range of metabolites are produced in plant in normal as well as under stressed condition which is a common characteristic of the plants. These metabolites are mainly antibacterial metabolites having broader range which are active against a concertia of Gram-negative and Gram-positive bacteria of agriculture and medicinal importance [22].

3.3 Determination of MIC and MBC of A. indica extract against Enterobacteriaceae

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extract against pathogenic strains of *Enterobacteriaceae* family e.g., *E. cloacae, E. coli, E. aerogenes, P. stuartii, K. pneumoniae* and *P. mirabilis* were determined using disc diffusion method. The concentration effect of the methanolic plant extracts was presented in Figure 1, showed that members of *Enterobacteriaceae* were susceptible to the extract. The MICs ranged from 40 to 100 mg/mL and MBCs ranged from 70 to 400 mg/mL (Table 3).

Table 3. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *A. indica* extract against bacteria

Strains	MIC	MBC
E. cloacae	70	400
E. coli	70	400
E. aerogenes	40	70
P. stuartii	70	400
K. pneumoniae	100	700
P. mirabilis	40	70

The results of MIC and MBC in the present study against *E. cloacae*, *E. coli, E. aerogenes*, *P. stuartii*, *K. pneumoniae* and *P. mirabilis* were consistent with the results of Penny et al.[23] and Ramesh et al.[24]. However, the results of the *P. granatum* extract were different in the range of MIC concentration. The reason of the variation might be due to the difference in bacterial species and strategies. For example, Cumin was found to be ineffective in against bacterial and the respective results was different from the results of Rolfsson et al. 2015 [25]. On the other hand, other findings revealed that a higher concentration of cumin extract was effective against bacteria of causing food spoilage [26]. Many researchers have studied the

efficacy of plant extracts and their active compounds as antimicrobial agents to control bacterial growth and food degradation [27].

4. CONCLUSIONS

The extract of *A. indica* was very effective as antibacterial agent against different pathogenic bacteria. The profile obtained by GC-MS showed that the extract of *A. indica* was rich with secondary metabolites in diverse form and in high concentration. The results revealed that the extract of *A. indica* could be a better and alternative source of antibacterial compound.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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