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Fractionation of Antimicrobial Compounds from *Acacia nilotica* Twig Extract Against Oral Pathogens

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Abstract: In the present study, the antimicrobial activity of fractions and sub-fractions of methanol extract of *Acacia nilotica* L. twig was done, and bioactive compounds were identified by GC-MS. Fractionation was done by column chromatography using different solvents, and their antimicrobial potential was checked by the agar well diffusion method. Minimum inhibitory concentration (MIC) was performed by the micro broth dilution method. Oral pathogens, including *Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans* were selected for the study. Results indicated that fraction (Fr-III) of *A. nilotica* methanol extract showed a significant zone of inhibition (ZOI) in the range of 14-15 mm against selected pathogens. Further sub-fraction, Fr-IIIf & Fr-IIIg of Fr-III exhibited maximum ZOI in a range of 38-40 mm at $P<0.05$. MIC of sample fractions was in the range of 80-210 µg/mL. GC-MS analysis represented that Piperidine,2,2,6,6-tetramethyl- was the major phytochemical in Fr-III. In the case of Fr-IIIg, n-Hexadecanoic acid was the main component, whereas behenic alcohol was in Fr-IIIf. Therefore, these bioactive compounds may be used as a potential therapeutic agent for oral health.

Keywords: *Acacia nilotica*; Column chromatography; Antimicrobial activity; Oral pathogens; GC-MS.

1. Introduction

Oral health is crucial often neglected part of our health, although its ignorance can cause many complications such as bleeding gums, toothache, oral sores, bad breath, dental caries, tooth sensitivity, tooth loss, oral cancer etc. [1]. According to the WHO report, oral infections affect around 3.5 billion people worldwide; among them, dental caries is the third most prevalent issue after cancer and cardiovascular diseases [2]. In recent studies, an individual's oral health status is also linked to heart diseases, gastrointestinal infections, utilization of nutrients, and diabetes [3]. An association between periodontal diseases and various types of cancer has also been evidenced [4]. The psychosocial impact of oral complications significantly reduces the quality of life. They have a profound impact on memory and cognition during the development and aging of the sufferer [5,6]. The most common cause of oral infections is the persistence of pathogenic agents such as *E. faecalis* are the most prevalent in the root canals of teeth [7] whereas excessive growth of *C. albicans* can elicit oral candidiasis which causes bad breath and biofilm formation [8]. In study *S. aureus* was the most common microorganism found in patients with gingivitis [9]. In recent years, the use of antibiotics, particularly

chlorhexidine, is resulting in the rapid growth of tolerance to antimicrobials [10]. Consequently, the search for new herbal products can provide better alternatives [11-14].

A. nilotica (Babool) has been used in ancient medicine for the treatment of several diseases, such as diarrhea, dysentery, hemorrhoid, pyorrhoea, abdominal aches, sore throat, etc diabetes, asthma, hypertension [15]. The use of the plant's twig (datun) is also a good way of dental care [16]. Various phytochemicals have been isolated from the *A. nilotica,* such as catechins, catechol, gallic acid, sitosterol, kaempferol, niloticane, D-pinitol, linoleic acid etc. [17]. Even its silver nanoparticle showed good antimicrobial activity [18].

Column chromatography is one of the most robust techniques for purifying novel bioactive [19]. It separates the compounds based on differences in their polarity, size, shape, and net charge [20]. The compounds in the plant extracts have different interaction abilities with the stationary phase (silica gel, which is polar) and a mobile phase (solvent). Henceforth they flow along with the mobile phase at different time intervals [21]. Finally, analysis of the eluted fractions can be done by using different techniques including TLC, HPLC, HPTLC, LC-MS, GC-MS etc. [22].

The use of gas-liquid chromatography (GLC) has been widely used in the field of medicine. In GLC, the sample is vaporized, and desorption is done by the flow of an inert gaseous mobile phase such as helium, nitrogen, argon, hydrogen, and carbon dioxide. The mobile phase does not interact with the analyses and only assist in their transport through the column. The investigation gets precipitated between a gaseous mobile phase and a liquid stationary phase [23]. Further, to identify these analytes, mass spectrometry is used. In this technique, ionization of the molecules occurs, and ions are then separated according to their mass-to-charge (*m/z*) ratios by mass-analyzer. The fragmented products are represented in the mass spectrum [24]. In our previous studies, of *A. nilotica* twig methanol extract showed significant antimicrobial activity among thirty-two plant extracts screened against the selected human oral pathogens [25]. Therefore as a continuation of the earlier work, in the current investigation, fractionation of *A. nilotica* methanol extract was done to screen antimicrobial compounds.

2. Materials and Methods

2.1. Collection of plant samples.

Plant samples (*A. nilotica* twig) were collected from Rohtak district, Haryana, India. The plant material was identified and authenticated by comparing the herbarium specimen (MDU 2601) available in the Department of Genetics, M. D. University, Rohtak.

2.2. Extract preparation.

15 g of *A. nilotica* shade-dried twigs were crushed to powder form. Afterward, it was dissolved in 150 mL of methanol and rotated for 2 days in incubator shaker. It was then filtered and lyophilized for fractionation.

2.3. Separation of phytochemical constituents by column chromatography.

The methanol extract of *A. nilotica* twig was fractionated and sub-fractionated by column chromatography. Glass column was packed with silica gel (100-200 mesh-Merck), which formed the stationary phase. Seven different solvents were used as mobile phase

including *n*-hexane, chloroform, ethyl acetate, methanol, distilled water, solvent 1(ethyl acetate : methanol : distilled water {70:29:1}), solvent 2(ethyl acetate : methanol : water : ethanoic acid {70:28:1:1}) were allowed to flow down through the column to elute sample fractions.

2.4. Antimicrobial activity against oral pathogens.

The zone of inhibition of the sample was determined against pathogens, namely *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 259323), and *Candida albicans* (ATCC 10231) using agar well diffusion method [26]. Minimum inhibitory concentration (MIC) was performed using a 96 well plate method.

2.5. Identification of the bioactive compounds.

Bioactive compounds present in the effective fraction and sub-fraction were identified by GC-MS at the Department of Genetics, MDU, Rohtak. The injection volume was 2 µL, and the carrier gas was helium at 1.22 mL/min. GC column oven temperature was 50°C for 3 min; 230°C for 5 min and finally 260°C for 18 min.

2.6. Statistical analysis.

Antimicrobial activity was performed in triplicate setups. The data from the zone of inhibition of different extracts were represented as mean ± standard error using ANOVA.

3. Results and Discussion

3.1. Antimicrobial activity.

In the present study, the zone of inhibition of various fractions of *A. nilotica* extract was ranged between 14-15mm against oral pathogens, as illustrated in Figure 1 (A, B & C) and Table 1.

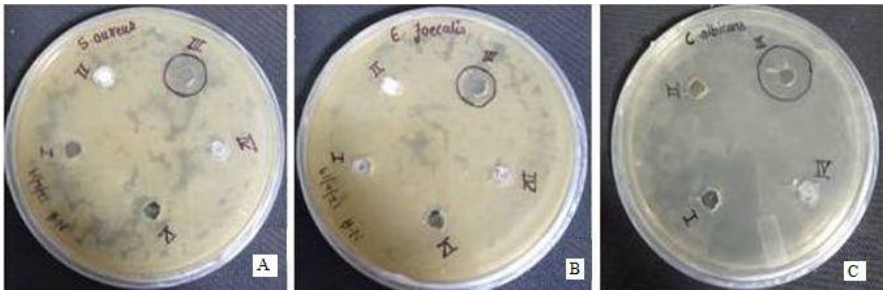

Figure 1. The ZOI of *A.nilotica* fraction against **(A)** *S. aureus* **(B)** *E. faecalis*, and **(C)** *C. albicans.*

Table 1. Zone of inhibition (in mm) of fraction and subfraction against oral pathogens.

Sample	*E. faecalis*	*S. aureus*	*C. albicans*
Fraction III	14 ± 1.28	15 ± 1.03	15 ± 0.78
Subfraction IIIf	38 ± 0.62	15 ± 1.42	20 ± 1.63
Subfraction IIIg	40 ± 1.48	40 ± 0.81	26 ± 1.51
Chlorohexidine (standard drug)	26 ± 0.43	25± 0.54	20 ± 0.34

Among all the fractions obtained by column chromatography, only Fr-III exhibited significant antimicrobial activity. In contrast, the remaining fractions did not show. Fr-III showed ZOI against *E. faecalis* (14 mm), *S. aureus* (15 mm), and *C. albicans* (15 mm). Sub

Fraction IIIf, IIIg demonstrated ZOI in the range of 15-40 mm at $p<0.05$ ($P\text{-value}_{0.10}$< F-crit $_{6.59}$), as depicted in Figure 2 (A, B & C) and Table 1.

Figure 2. The ZOI of *A.nilotica* sub-fraction against (A)*S. aureus* (B) *E. faecalis* and (C) *C. albicans.*

Fr-IIIg was most effective against *S. aureus*, *E. faecalis*, and *C. albicans* with ZOI of 40, 40, and 26 mm, respectively. Whereas Fr-IIIf showed ZOI against *S. aureus* (15 mm), *E. faecalis* (38 mm), and *C. albicans* (20 mm). Furthermore, the lowest MIC was shown by Fr-IIIg against *E. faecalis* at 80 µg/mL as represented in Figure 3.

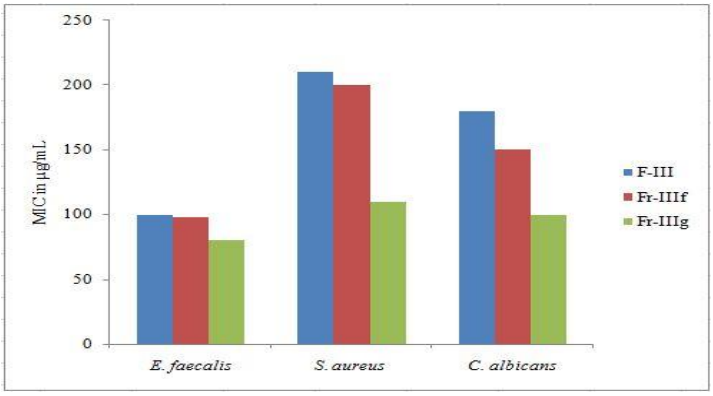

Figure 3. MIC of *A. nilotica* twig fraction against oral pathogens.

In the earlier report, also different parts of *A. nilotica* showed significant antimicrobial activity against *Staphylococcus aureus*, *S. mutans*, *E. faecalis* and *C. albicans*, including its leaf [27,28], fruit pod [29], bark [30], twig [31]. Moreover, the selection of solvents in increasing polarity order proves the effect of polarity on the extraction of different phytochemicals [32].

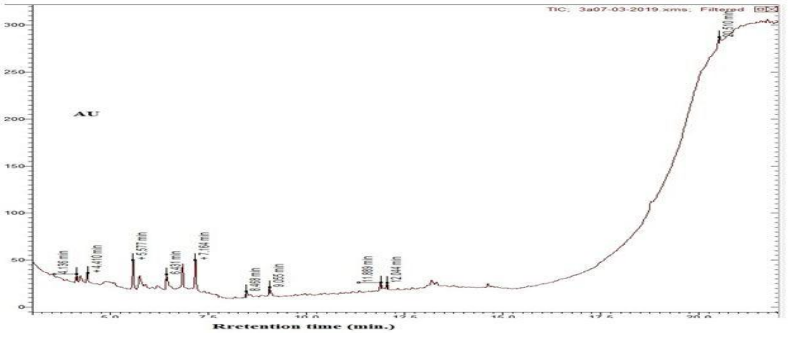

Figure 4. GC-MS spectra for fraction III of *A. nilotica* twig.

3.2. GC-MS analysis.

Fraction III, Fr-IIIf, and Fr-IIIg were processed for GC-MS analysis. Different peaks represent the compound with their retention time and area percentage, as illustrated in Figures 4, 5 & 6, and Table 2, 3 & 4.

Table 2. Chemical profile of fraction III of *A. nilotica* twig using GC-MS.

S. No.	RT	Name	Area percentage
1	4.141	Ethanol,2-(trimethylsilyl)-	4.43
2	4.233	1-Methyl-2-tert-butylpyrrole	5.76
3	4.418	Pyridine,2,4,6-trimethyl-	6.25
4	5.573	4-Pipendinone,2,2,6,6-tetramethyl-	14.70
5	5.742	1,3,5-Triazine, hexa hydro-1,3,5-tris (2,2,6,6-tetramethyl-4-pipendinyl)-	9.25
6	6.435	Spiro[2,5]octane-1,1-dicarbonotrile,2-methyl-	7.83
7	6.834	Silane,dimethoxydimethyl-	13.83
8	7.158	4-Pipendinone,2,2,6,6-tetramethyl-,oxime	14.94
9	8.466	Benzene,(1,2,2-trimethoxyethyl)-	2.27
10	9.051	Phenol,2,4-bis(1,1-dimethylethyl)-	5.85
11	11.883	Ethanone,2,2-dimethoxy-1,2-diphenyl	5.17
12	12.037	Hexadecanoic acid, methyl ester	5.23
13	20.514	9-Octadecenamide,n-cyclopropyle-	4.43

A number of phytochemicals present in the fraction samples are bioactive. Fr-III contain the highest area of 4-4-Pipendinone,2,2,6,6-tetramethyl- and its oxime demonstrated strong antioxidant activity. The compounds are alkaloid in nature and also abundantly found in *Piper nigrum* L [33]. Phenol,2,4-bis(1,1-dimethylethyl)- a catechol, reported antifungal activity against many phytopathogens [34]. Presence of silane, dimethoxydimethyl- peak in the fraction can be due to GC injector, column, or septal bleeding as highly acidic or basic compounds in sample mixture can do this [35].

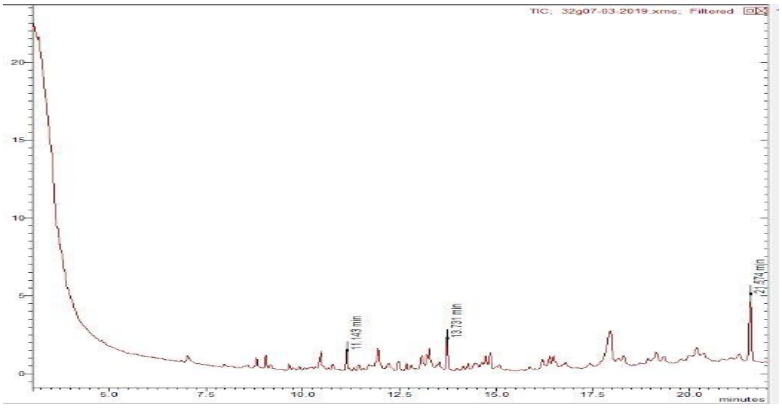

Figure 5. GC-MS spectra for Fr-IIIf of *A. nilotica* twig.

Table 3. Chemical profile of Fr-IIIf of *A. nilotica* twig using GC-MS.

S. No.	RT	Name	Area percentage
1	11.050	n-Nonadecanol-1	13.050
2	13.738	Behenic alcohol	21.866
3	21.570	Dimethyl(octadecyloxy)(octyloxy)silane	65.083

Furthermore, sub-fraction Fr-IIIf contains n-docosanol or behenic alcohol, showed significant antibacterial activity against *Klebsielia pneumonia* and *Staphylococcus aureus* [36].Another fatty alcohol found in the fraction is n-Nonadecanol-1, which exhibited a strong cytotoxic effect against human cancer cell lines viz. MCF-7 and HeLa. The compound is also a major component present in the essential oil of *Ceratonia siliqua* and *Heracleum thomsonii* [37,38].

Fr-IIIg contains many fatty acids, including n-hexadecanoic acid, heptanoic acid, nonanoic acid, oleic acid. In an earlier study, fatty acids and their esters displayed strong antimicrobial activity against oral pathogens [39].

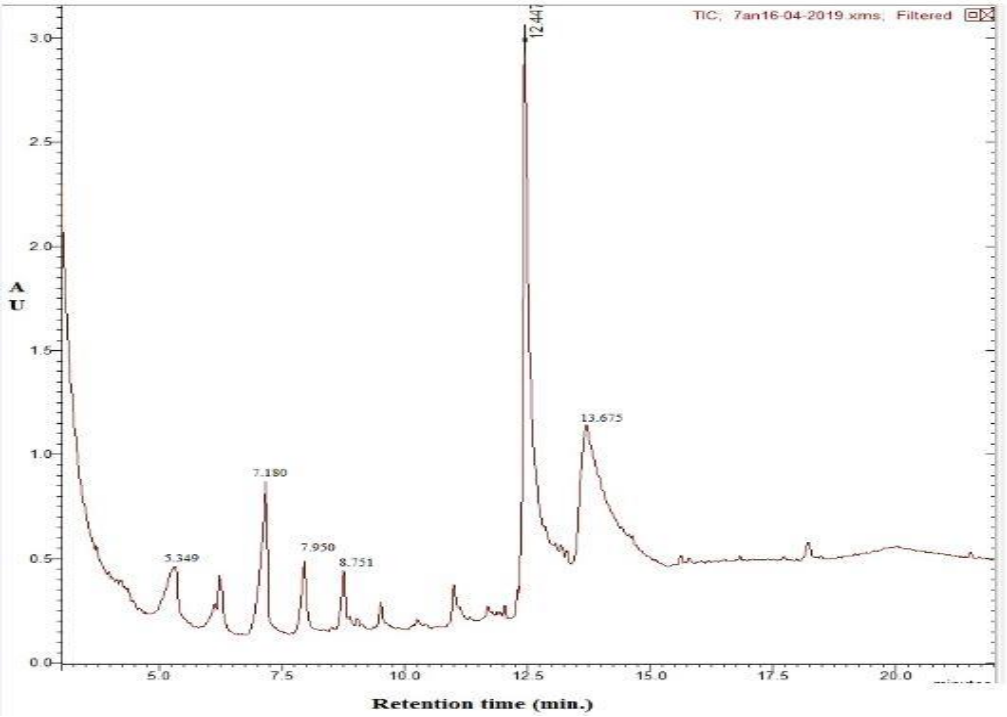

Figure 6. GC-MS spectra peaks for Fr-IIIg of *A. nilotica* twig.

Table 4. Chemical profile of Fr-IIIg of *A. nilotica* twig using GC-MS.

S.No.	RT	Name	Area percentage
1	5.349	Heptanoic acid	5.43
2	7.180	Nonanoic acid	9.76
3	7.950	Decanoic acid	2.25
4	8.751	Undecanoic acid	1.78
5	12.444	n-Hexadecanoic acid	55.54
6	13.675	Oleic acid	25.24

Bacteriocidal effects of hexadecanoic acid, which is a major compound in the sub-fraction, were elucidated against *S. aureus* by increasing membrane fluidity and disruption in the proton motive force [40,41]. In many previous studies, it also showed various pharmacological activities such as anti-inflammatory [42], antioxidant[43], hypocholesterolemic [44]. The n-hexadecanoic acid demonstrated significant cytotoxic activity against human colorectal carcinoma cells with IC_{50} value of 0.8 µg/mL [45]. Heptanoic acid demonstrated bactericidal properties for entero-toxigenic *Escherichia coli* [46]. Nonanoic acid, which is a saturated fatty acid, exhibited inhibition of *C. albicans* growth at lower concentrations [47]. Oleic acid has potent antibacterial potential against *S. typhimurium* and *S. aureus* [48]. Decanoic acid, also known as capric acid, showed growth inhibition of *E. faecalis* [49].

4. Conclusions

Column Chromatography fractions of *Acacia nilotica* twig's methanol extract showed the inhibitory activity against selected oral pathogens. GC-MS identified many bioactive compounds that can be responsible for the antimicrobial activity and could pave away in the production of proficient herbal toothpaste, endodontic irrigants, mouth fresheners, mouthwashes, dental gels, etc. However, depending on the nature of research, desire natural products can be further purified using fractional crystallization, distillation, sublimation, thin layer chromatography, high-performance liquid chromatography, etc.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

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