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IN VITRO ASSESSMENT OFANTIMICROBIAL PROPERTIES OF PETROLEUM ETHER AND AQUEOUS-METHANOL EXTRACTS, AND SOME FRACTIONS OF *NICOTIANA GLAUCA* FLOWERS, LEAVES, STEMS AND ROOTS

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ABSTRACT

The main objective of this study deals with the preparation of petroleum ether and aqueous-methanol extracts from flowers, leaves, stems and roots of *Nicotiana glauca* plant as well as chloroform, ethyl acetate and n-butanol fractions from Albaha region, Kingdom of Saudi Arabia. Furthermore, the obtained extracts and fractions were assessed for their antimicrobial activity against four bacterial (two Gram-positive and two Gram-negative) and two fungal reference strains, using disc diffusion method. The results of the study show that petroleum ether extract exhibited the highest activity against all the tested bacterial strains as compared to aqueous-methanol extract and among all fractions, ethyl acetate fraction possess a good sensitivity

through the different strains. Tested for their antifungal properties, all tested extracts and fractions are inactive against *Aspergillus flavus* but they exhibited in some cases a moderate activity against *Candida albicans* as compared to Amphotericin B, used as positive control. Thereby, our results justify the possibility to use the obtained extracts and fractions in the treatment of some bacteria and fungi infections.

Keywords: *Nicotiana glauca,* extracts, fractions, antimicrobial properties

INTRODUCTION

Herbs and natural plant products have been available for centuries and are intimately linked and applied by human as curative methods of many fatal diseases throughout history [1]. According to the World Health Organization (WHO), about 80% of the world's population uses traditional medicine through plant extracts, essential oils and their bioactive molecules for the primary health care [2]. In addition, natural products and their derivatives represent more than 60% of all drugs used clinically in the world and in particular for the treatment of type II diabetes, antitumor and anti-infectious diseases. Their therapeutic properties are due in large part to the presence of secondary bioactive compounds [3, 4].

Nicotiana glauca plant known by its common name tree tobacco is an ornamental, evergreen, bluish green, erect, slender, sparsely-branched perennial, a fast and soft-woody shrub to small tree plant, belongs to Solanaceae family [5]. A survey of literature showed that *Nicotiana glauca* extracts provide a large broad spectrum of biological properties and influence a number of physiological functions. It was found that the principle isolated alkaloids, anabasine from *Nicotiana glauca* have an arthrogrypotic congenital on piglets and found significant result [6]. It was reported that extracts can be served as antioxidant [7-10], antimicrobial [10, 11], antifungal [12, 13], cytotoxic activities [11] anti-inflammatory activity [8,11], larvicidal [14]. Isolation of Scopoletin (6-methoxy-7-hydroxycoumarin) from *Nicotiana glauca* extracts was found to have an hepatoprotective effect [15], spasmolytic [16], antioxidant [17], anti-proliferation [40, 41], and anti-tumor activities [18]. It was demonstrated that non-boiled *Nicotiana glauca* aqueous extract from leaves reduced total serum bilirubin level while that of the boiled and non-boiled aqueous extracts from flowers were non effective [19].

The desire to turn to herbal treatments comes largely from the risks of modern medicine. In fact, it is well-known that a part of

conventional medicines present risks of accidents that could compromise the health or life of patients. Also, the inadequate and abusive use of synthetic antioxidants as well as increasing proliferation of multi-drug resistant bacteria in human health is being a major problem because of potential toxicological and cancer risks as well as the limited number of antibiotics efficiency and their discriminate uses [20]. The drug resistant of pathogen microbes have further delayed the treatment of infectious diseases [21]. Till now, one way to combat and reduce the resistance to antibiotics against pathogenic strains is to use plant extracts and their bioactive compounds for providing new antimicrobial agent from natural molecules that not are accessible by other routes which is remains a promising alternative and a good challenge for the pharmaceutical industries[4].

The main objective of this study deals with the preparation of petroleum ether and aqueous-methanol extracts from *Nicotiana glauca* (flowers, leaves, stems and roots), their fractionation and to investigate their antimicrobial activity. This may lead to the discovery of an alternative form based on natural products in order to combat the resistance of certain microbes against the antibiotics currently used.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used were purchased from Sigma-Aldrich (reagent grade, Germany) and Merck Co. (Germany), Loba (India). They are used directly without further purification. GC-MS were recorded on Thermo scientific Xcalibur.

Plant material

Nicotiana glauca plant (leaves, stems, flowers and roots) was collected from Albaha region, Kingdom of Saudi Arabia and was authenticated by Dr. Haider Abd Algadir, chemo taxonomist, department of Biology, Faculty of Science, Albaha University, Albaha.

Extraction and fractionation:

The plant parts flowers (F), leaves (L), stems (S) and roots (R) were shade dried for three weeks and coarsely powdered to get 750 (F), 750(L), 750(S) and 300(R) g from parts powder. This powder materials were extracted with petroleum ether (40-60) in a Soxhlet apparatus (500 mL), the petroleum ether extracts were evaporated under reduced pressure using rotatory evaporator to get11 (F), 29(L), 8(S) and 4(R) g crudes extract.

The defatted plant materials 735(F), 720(L), 740(S) and 295(R) g, were soaked in (4.0 L) of aqueous-methanol: water (7:3) for two week and filtered, again the plant material

was extracted in similar manner two times. The combined methanol extracts were dried by evaporating the solvents using rotary evaporator to get 35(F), 55(L), 29(S) and 19(R) g of aqueous-methanolic crude extracts. After that, the aqueous-methanol extract was partitioned with chloroform (100 mLX3) to get 3.5(F), 5.0(L); 2.5(S) and 1.5(R) g, ethyl acetate (100 mLX3) to get 3.0(F), 6.5(L), 3.0(S) and 2.5(R) g and finally n-butanol (100 mLX3) to get4.0(F),7.5(L), 3.5(S)and 2.5(R) g crude fractions, respectively.

Antimicrobial activity

All the antimicrobial studies were performed at National Research Centre, Egypt. The antimicrobial activity of the tested extracts was determined using a modified Kirby-Bauer disc diffusion method [22]. 100 µl of the test bacteria and fungi were grown in 10 mL of fresh media until they reached a count of approximately 10^7 colony forming unit (cfu)/mL for bacteria and10^5 colony forming unit (cfu)/mL for fungi [23]. 100 µl of microbial suspension was spread onto Meuller-Hinton agar plates. Standard discs (200 µg) of Ampicillin (antibacterial agent) and Amphotericin B (antifungal agent) served as positive controls. The blank paper discs with a diameter of 8.0 mm were impregnated 10µL of tested extracts of the stock solutions (100 mg/mL) and filter discs impregnated with 10 µl of solvent (distilled water, chloroform, DMSO) were used as a negative control and were placed on to the plates. The plates inoculated with filamentous fungias *Aspergillus flavus* incubated at 25^{o}C for 48 hours; Gram positive bacteria (*Staphylococcus aureus, Bacillus subtilis*); and Gram negative (*Escherichia coli*, *Pseudomonas aeuroginosa*) bacterial strains were incubated at 35 – 37 ^{o}C for 24 - 48 hours and yeast as *Candida albicans* incubated at 30^{o}C for 24 - 48 hours and, then the diameters of the inhibition zones were measured in millimeters [22].

RESULTS

Preparation of plant extracts and fractions

The plant materials from different parts (leaves, stems, flowers and roots) of *Nicotiana glauca* were extracted with petroleum ether then aqueous-methanol extracts. The concentrated extracts (petroleum ether and aqueous-methanol) were further subjected to partial fractionation with different solvents.

The choice of the solvent is a crucial step for the extraction of plant materials and relies mainly on two conditions: It must be non-toxic and removed easily by evaporation. In our case, we are opted for petroleum ether

and methanol. Extraction with petroleum ether as less polar solvent used to extract non-polar chemicals and intended to entrain fats as well as lipophilic substances. Methanol has a polarity index of 5.1 is widely used to extract a large range of phytochemical compound, especially polar compounds but certain group of non-polar compounds that are fairly soluble in methanol if not readily soluble and therefore may be extracted. Also, it was used because of its low boiling point which helps to evaporate the filtration within a short time.

The fractionation was done with chloroform which used to obtain an extract rich in moderately polar compounds. After, extraction with ethyl acetate was chosen to obtain a polar compound-rich extract. Finally, n-butanol extraction was opted to obtain an extract rich in very polar compounds.

***In vitro* antimicrobial activity assessment**

All extracts and fractions (chloroform, ethyl acetate and n-butanol) of *Nicotiana glauca* parts (flowers, leaves, stems and roots) were screened for their antimicrobial activity against four bacterial (two Gram-positive and two Gram-negative)and two fungal reference strains, using disc diffusion method. The antibacterial activity of extracts, fractions and their known antibacterial agent, ampicillin and antifungal agent, amphotericin B, was determined by the presence or absence of the inhibition zone diameter (IZD) around the discs. As shown (**Table 1** and **2**), all extracts and fractions have antibacterial activity against all tested microorganisms except for PEEL which is inactive, and weak or even no active against tested fungi.

The results presented in **Table 1**for petroleum ether extract revealed that *Nicotiana glauca* PEEF is more susceptible to different strains than PEEL, PEES and PEER as compared to the positive control, Ampicillin with the strongest activity was ascribed to *Staphylococcus aureus* (IZD = 16 *vs.* 21 mm) followed *Bacillus subtilis* (IZD = 14 mm *vs.* 26 mm), *Pseudomonas aeruginosa* (IZD = 13 mm *vs.* 26 mm) and *Escherichia coli* (IZD = 12 mm *vs.* 25 mm). We note the higher resistance of the tested bacteria to the PEEL.

As shown for aqueous-methanol extract (**Table 1**), all *Nicotiana glauca* extracts demonstrated various antimicrobial powers against all the tested bacterial strains with the potent inhibitory activity was allowed to *Staphylococcus aureus* for flowers and leaves (IZD = 12 mm *vs.* 21 mm), followed by stems and roots (IZD = 9 mm *vs.* 21 mm) in comparison with *Bacillus subtilis,*

Escherichia coli and *Pseudomonas aeruginosa* for AMEL, AMES and AMER. AMES and AMER exhibited similar antibacterial activity against *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa.*

The reported results of chloroform fraction(**Table 1**) showed that all fractions were active against all strains as compared to the positive control ampicillin with an interesting activity was found against *Staphylococcus aureus* for CFL, CFS and CFR (IZD = 15 *vs.* 21) respectively, followed by CFF (IZD = 14 *vs.* 21). Also, CFS and CFR inhibit approximately with the same way the growth *Bacillus subtilis* (IZD =14 mm) *Staphylococcus aureus* (IZD =15 mm), *Escherichia coli* (IZD =16 mm) and *Pseudomonas aeruginosa* (IZD = 14-15 mm).

In ethyl acetate fraction, EAFF, EAFL, EAFS and EAFR showed the most potent activity against *Staphylococcus aureus* (IZ = 15-16 mm), followed by *Bacillus subtilis* (IZ = 14-16 mm), *Escherichia coli* (IZ = 14-15 mm) and *Pseudomonas aeruginosa* (IZ = 14-15 mm) as compared to the standard, ampicillin, with IZD = 21 mm, 26 mm, 25 mm and 26 mm, respectively (**Table 1**).

For n-butanol fraction, the greatest antibacterial activity was obtained against *Staphylococcus aureus* (IZD=13mm) for BFF, BFL and BFS followed by BFR (IZD=13mm) against *Bacillus subtilis* as compared to Ampicllin, with IZD=21 and 26mm, respectively. We note the mild sensitivity of roots against all tested bacteria (**Table 1**).

No antifungal activity was observed (**Table 2**) independently of the used *Nicotiana glauca* plant parts, against *Aspergillus flavus* and *Candida albicans* for two extracts and for different fractions (chloroform, ethyl acetate and n-butanol), except for *Candida albicans* in the case of CFL (IZD = 11mm), CFS (IZD = 10 mm), CFR (IZD = 9 mm), EFS (IZD = 9 mm) and EFR (IZD = 10 mm).

Table 1: Inhibition zone diameter of *Nicotiana glauca* extracts and fractions against bacterial strains

Samples	IZD (mm)			
ATCC	Bacterial species			
	Gram positive		Gram negative	
ATCC	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*
ATCC	6051	12600	11775	10145
PEEF	14	16	12	13
PEEL	0	0	0	0
PEES	10	11	11	12

PEER	**10**	**10**	**11**	**10**
AMEF	**11**	**12**	**10**	**11**
AMEL	**11**	**12**	**11**	**11**
AMES	**9**	**9**	**9**	**9**
AMER	**9**	**9**	**9**	**9**
CFF	**12**	**14**	**12**	**13**
CFL	**13**	**15**	**14**	**14**
CFS	**14**	**15**	**16**	**14**
CFR	**14**	**15**	**16**	**15**
EAFF	**16**	**16**	**14**	**15**
EAFL	**15**	**16**	**14**	**15**
EAFS	**16**	**15**	**15**	**15**
EAFR	**14**	**16**	**15**	**14**
BFF	**13**	**13**	**10**	**10**
BFL	**12**	**13**	**10**	**12**
BFS	**12**	**13**	**11**	**12**
BFR	**10**	**10**	**10**	**10**
Ampicillin	**26**	**21**	**25**	**26**

Petroleum ether extract of flowers (PEEF), petroleum ether extract of leaves (PEEL), petroleum ether extract of stems (PEES), petroleum ether extract of roots (PEER), aqueous-methanol extract of flowers (AMEF), aqueous-methanol extract of leaves (AMEL), aqueous-methanol extract of stems (AMES), aqueous-methanol extract of roots (AMER), chloroform fraction of flowers (CFF), chloroform fraction of leaves (CFL), chloroform fraction of stems (CFS), chloroform fraction of roots (CFR), ethyl acetate fraction of flowers (EAFF), ethyl acetate fraction of leaves (EAFL), ethyl acetate fraction of stems (EAFS), ethyl acetate fraction of roots (EAFR), n-butanol fraction of flowers (BFF), n-butanol fraction of leaves (BFL), n-butanol fraction of stems (BFS), n-butanol fraction of roots (BFR)

Table 2: Inhibition zone diameter of *Nicotiana glauca* extracts and fractions against fungal strains

Samples	**IZD (mm)**	
ATCC	**Fungal species**	
ATCC	***Aspergillus flavus*** **Link**	***Candida albicans*** **7102**
PEEF	**0.0**	**0.0**
PEEL	**0.0**	**0.0**
PEES	**0.0**	**0.0**
PEER	**0.0**	**0.0**
AMEF	**0.0**	**0.0**
AMEL	**0.0**	**0.0**
AMES	**0.0**	**0.0**
AMER	**0.0**	**0.0**
CFF	**0.0**	**0.0**
CFL	**0.0**	**11**
CFS	**0.0**	**10**

CFR	**0.0**	**9**
EAFF	**0.0**	**0.0**
EAFL	**0.0**	**0.0**
EAFS	**0.0**	**9**
EAFR	**0.0**	**10**
BFF	**0.0**	**0.0**
BFL	**0.0**	**0.0**
BFS	**0.0**	**0.0**
BFR	**0.0**	**0.0**
Amphotericin B	**17**	**21**

Petroleum ether extract of flowers (PEEF), petroleum ether extract of leaves (PEEL), petroleum ether extract of stems (PEES), petroleum ether extract of roots (PEER), aqueous-methanol extract of flowers (AMEF), aqueous-methanol extract of leaves (AMEL), aqueous-methanol extract of stems (AMES), aqueous-methanol extract of roots (AMER), chloroform fraction of flowers (CFF), chloroform fraction of leaves (CFL), chloroform fraction of stems (CFS), chloroform fraction of roots (CFR), ethyl acetate fraction of flowers (EAFF), ethyl acetate fraction of leaves (EAFL), ethyl acetate fraction of stems (EAFS), ethyl acetate fraction of roots (EAFR), n-butanol fraction of flowers (BFF), n-butanol fraction of leaves (BFL), n-butanol fraction of stems (BFS), n-butanol fraction of roots (BFR)

DISCUSSION

Interestingly, the results showed that petroleum ether extract proved to be the most effective against all the tested bacterial strains than aqueous-methanol extract, except for PEEL, which is remains inactive (**Table 1**). Also, we notice the strong resistance of the different strains to PEEL in contrast to MEL. Regarding the antimicrobial activity of the various fractions (chloroform, ethyl acetate and n-butanol), it is found that the ethyl acetate fraction is more sensitive to the different strains (**Table 1**).

More recently, Al-Robai (results not published till now) found that all *Nicotiana glauca* extracts were inactive against *Escherichia coli* and *Salmonella typhi* while leaves and root bark extracts showed remarkable activity towards *Staphylococcus aureus*when compared with the used antibiotic standards, which is agree with this work (*Staphylococcus aureus)*. No antibacterial activity was found for flowers extract.

The differential sensitivity of Gram positive and Gram negative bacteria to different extracts and fractions may be explained by the morphological differences between the microorganisms cell wall including cell envelope, that be expressed by lipoprotein and lipopolysaccharide, and which plays the role of a barrier to the antibacterial substances [20]. The difference in morphological structure between the used

microorganisms and the water solubility of the tested extracts and fractions may be also taken account [24].

The absence of antifungal activity is probably due to the presence of active ingredient in *Nicotiana glauca* extract and fractions in very low quantities, which needed the use of large amounts of crude extracts and fractions. Therefore, we suggest that they may be act in an indirect way; the active ingredient may exist as a precursor, which requires activation in the body by *in vivo* unknown mechanism [20]. Also, the difference in strains behavior with different extracts and fraction may be due to their intrinsic properties that are related to their surface cells permeability [25]. It was reported that the endophytic bacteria (*Alcaligenes faecalis* and *Bacillus cereus*) isolated from *Nicotiana glauca* possess an antifungal potential towards *Fusaium oxysporum* f. sp. *Lycopersici* [13, 26, 27]. Al-Robai (2019) (results not published till now) reported that flowers extract exhibited very powerful antifungal activity against *Candida albicans* and *Aspergillus niger*.

CONCLUSION

In summary, the evaluation of the *in vitro* antibacterial activity provides the effectiveness of petroleum ether extract against the tested bacteria than aqueous-methanol extract. Fractionation was manifested generally by an increase in the antimicrobial activity as compared to both extracts with ethyl acetate fraction was expected to be the most active. In addition, the used extracts and fractions were tested for their antifungal activity and the results show no activity against *Aspergillus flavus* for all different plant parts; however *Candida albicans* was moderately sensitive with CFL, CFS, CFR, EFS and EFR and remains inactive with the other extracts.

These differences could be attributed to the diversity in chemical composition of extracts and fractions. The obtained fractions as well as PEEF provide an important tool for the treatment of infectious diseases caused by resistant microorganisms. Therefore, it will be interesting to elucidate their major active compounds and to demonstrate their mode of action against resistant bacteria, especially *Staphylococcus aureus*.

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