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Studies on total phenolics, total flavonoids and antimicrobial activity from the leaves crude extracts of neem traditionally used for the treatment of cough and nausea

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ABSTRACT

The objective of this work is to prepare different crude extracts from the leaves of neem through maceration method and determine their total phenolics, flavonoids and antimicrobial activity by established methods. The different crude extracts were prepared solvents by maceration method using solvents of different polarities. Total phenolics and total flavonoids contents were determined by using UV–visible spectroscopy method. The antimicrobial activity of different crude extracts from the leaves of neem was determined by disc diffusion method against food borne pathogenic bacterial strains *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Vulgaris*. Amoxicillin was used as a positive control. The content of total phenolics of different leaves crude extracts was in the range of 20.80–107.29 mg/100 g of powder crude extracts. The content of total flavonoids of different leaves crude extracts was in the range of 61.50–529.50 mg/100 g powder samples. All crude extracts from neem by maceration method at different working concentrations did not show any potential antimicrobial activity. In conclusion, our results of all crude extracts prepared by solvents of different polarities do not support their use as medicine for treating cough and nausea due to high content of total phenolics and flavonoids.

1. Introduction

Azadirachta indica tree belongs to the Meliaceae family and it originates from Pakistan, India and Thailand (Sithisarn and Gritsanapan, 2010). Locally known as Azad-darakhul-hind, and neem in English. *Azadirachta* is a fast growing tree with wide and spreading branches. All-year-round, neem leaves are mixed, both young and matured; the mature leaves are bright green in color. whereas the young ones are reddish to

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purple. Normally, as a foliage, the neem leaves consists of Petiole, lamina, and base which attaches the leaves to the stem and may bear two lateral small leaves like structures called stipules (Norten, 1996) (Fig. 1).

Neem leaves are regarded by Ayurvedic healers as an effective internal cleanser. Neem leaves have a powerful purifying effect on the blood and help cleanse the liver and skin of toxins. Neem leaf tea with a dash of honey can help soothe a dry irritated throat (Maragathavalli et al., 2012).

Traditionally, the neem is very important medicine in Indian culture for the treatment of different aliments. It is one of the main sources of many therapeutic agents (Maragathavalli et al., 2012). In the traditional Thai medicine system, the leaves and flowers are used as a tonic and in the treatment of fever (Mohashine et al., 2009). Moreover, leaves and seed oils constituent an important medicine for relieving psoriasis itch and lesions (Conrick, 2001). Another part of neem is the fruit which is used as anthelmintic and for the treating of hemorrhoids and abnormal urination (Chaturvedi et al., 2011). Remarkable about the neem tree is that it produces allergies and is therefore traditionally preferred over others for medicinal use (Siswomihardjo et al., 2009).

In different medicinal systems, it is used widely as an alternative therapeutic tool for the prevention or treatment of many diseases (Kiranmai et al., 2011). Each part of neem tree has several medicinal values to treat a wide range of human disorders such as antiseptic, diuretic, cough, nausea, vomiting, fever and peptic ulcer (Iranmai et al., 2012). The juice of this plant is used traditionally for the treatment of gastrointestinal disease (Iranmai et al., 2012). The leaves are used traditionally by Indian people for the treatment of chicken pox sleep (Asif, 2012).

Extensive work was done on the analysis of chemical constituents of neem crude extracts. Many bioactive components were isolated and identified from neem crude extracts such as azadirachtin, salannin, meliantriaol and nimbin (Kokate et al., 2010). Among them, the most active ingredient reported is azadirachtin (Kokate et al., 2010; Asif, 2012). These chemical constituents belong to the classes: beta-sitosterol, stigmasterol and limonoids (Kokate et al., 2010; Asif, 2012; Hismath et al., 2011). Also the other tri and tetra cyclic compounds were isolated from this plant such as sulphides, flavonol glycosides. Nimaton, quercetin, myrecetin, kaempferol (Kokate et al., 2010; Asif, 2012; Hismath et al., 2011).

Fig. 1 – Image of leaves of neem.

Traditionally, Omanis use the whole plant for the treatment of fever (Kokate et al., 2010; Asif, 2012; Hismath et al., 2011). The literature search reveals that no scientific data is available in Oman on total phenol, flavonoids and antimicrobial activity of neem leaves. Therefore, the main objectives of this present study are to determine the total phenolics, total flavonoids content and evaluation antimicrobial activity from the leaves crude extracts collected locally.

2. Materials and methods

2.1. *Materials and chemicals*

Solvents like acetone, butanol, chloroform, ethyl acetate, methanol, ethanol, hexane dichloromethane, dimethyl sulphoxide (DMSO) and gallic acid and quercetin were collected from Sigma–Aldrich company, Germany. Other chemicals such as sodium hydroxide and aluminum chloride were obtained from Philip Harris-England. Folin–Ciocalteu reagent and sodium nitrate for total phenolics were obtained from Scharlau, Spain. UV–visible spectrophotometer (UV–visible) was from Jasco Company, Japan.

2.2. *Bacterial strains*

The food borne pathogenic bacterial strains such as *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* and (*P. vulgaris*) were collected from the Department of Biological Sciences, School of Arts and Sciences, University of Nizwa, Sultanate of Oman.

2.3. *Plant sample*

The leaves samples were collected from Al-Amerat, Muscat, Sultanate of Oman on 8/2/2014 at 3 pm. The collected samples were transported at home, Nizwa for processing. The dry leaves samples were transported to the Natural Product Lab, School of Pharmacy, College of Pharmacy and Nursing, University of Nizwa, Oman for powder. The plant was identified by Botanist.

2.4. *Preparations of samples*

The samples were washed to remove dust and then dried in an oven at a temperature not exceeding 40 °C. The dried samples were then powdered by using grinding machine.

2.5. *Extraction procedure*

The powdered samples (100 g) prepared above were suspended in methanol (250 ml) and the mixture held for 3 days at room temperature. Then the mixture was filtered using a Bruckner Funnel and the methanol solvent evaporated from the filtrate in a round-bottom flask using a rotary evaporator. The methanol free crude extract was defatted in distilled water (150 ml) then transferred to separatory funnel for

Table 1 – Total phenolics content of different crude extract from the leaves of neem.

Crude extracts	Total phenolics (mg/g dry crude extract)
Hexane	22.77
Chloroform	20.80
Ethyl acetate	106.56
Butanol	107.29
Methanol	43.57
Water	51.02

extraction. The samples were extracted using different solvents with increasing polarity such as hexane, chloroform, ethyl acetate and butanol, each being added twice (30 ml and 20 ml) to complete the extraction.

2.6. *Determination of total phenolics content*

The total phenolics content was determined by the Folin–Ciocalteu reagent (FCR) method. For the assay, 10 ml of Folin–Ciocalteu reagent (FCR) and 6% Na_2CO_3 solution, both in distilled water, were prepared. Each sample of crude extract was suspended in 4 ml of methanol and aliquots of 200 μl of each were dispensed in separate tubes. 1.5 ml of 10% FCR was added to each tube followed by incubation for 5 min in the dark. Then 1.5 ml 6% sodium carbonate was added to each tube and shaken by hand to mix. All the tubes were incubated for 2 h in the dark and finally absorbance was measured by UV–visible spectroscopy at 760 nm wavelength (Hossain et al., 2013).

2.7. *Determination of total flavonoids content*

Each sample of the leaves crude extract (4 mg) was suspended in methanol (4 ml). 0.25 ml of each suspension was transferred to a tube followed by addition of 1.25 ml distilled water and 5% $NaNO_3$. After 6 min, 150 μl 10% aluminum chloride were added and the mixture left for 5 min in the dark. This was followed by adding 0.5 ml 5% sodium hydroxide and 0.275 ml water and measurement of absorbance by UV–visible spectroscopy at 450 nm wavelength (Hossain et al., 2013).

2.8. *Antimicrobial activity*

For testing antimicrobial activity, each crude extract (10 mg) was mixed with 5 ml DMSO (Hossain et al., 2014a,b) and

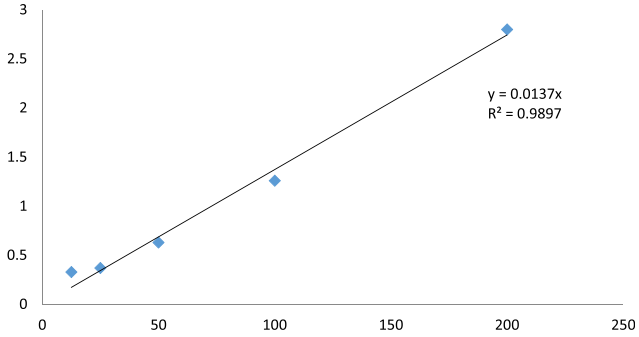

Fig. 2 – Gallic acid standard calibration curve.

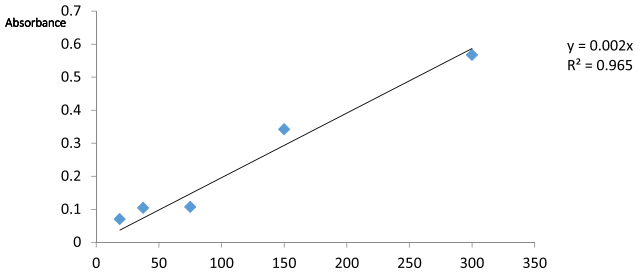

Fig. 3 – Quercetin standard calibration curve.

serially decreasing concentrations were made (2 mg/ml, 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml). Amoxicillin (1 mg/ml in DMSO) was used as a standard control. Each concentrations was tested by the agar diffusion method against 1 g (+) bacteria *S. aureus* and 3 g (–) bacteria *E. coli* and *P. aeruginosa* and *P. vulgaris*. Filter paper discs (Whatman No. 41, 6 mm diameter) were impregnated with each crude extract and place on the inoculated agar. All the plates were incubated at 37 °C for 24 h. The evaluation for antibacterial activity was measured the diameter of zones of inhibition against the tested food borne pathogenic bacteria strains. Each method in this experiment was replicated three times.

3. Results

The leaves powder samples were used for the extraction with methanol by maceration method. After complete extraction, methanol was evaporated by rotary evaporator and defatted with water. Different solvents such as hexane, chloroform, ethyl acetate and butanol were used for extraction with increasing polarities.

3.1. *Total phenolics content*

All crude extracts from the leaves of neem were used for the determination of total phenolics content by using Folin–Ciocalteu reagent (Hossain et al., 2013). The results of total phenolics content by using Folin–Ciocalteu reagent are presented in Table 1. From Table 1, the highest amount of total phenolics was obtained in butanol crude extract and the lowest was chloroform crude extract. The order of total phenolics was butanol > ethyl acetate > water > methanol > hexane > chloroform. The gallic acid was used for a standard calibration curve (Table 1 and Fig. 2).

Table 2 – Total flavonoids content of different crude extract from the leaves of neem.

Crude extracts	Total flavonoids (mg/100 g dry powder)
Hexane	491.0
Chloroform	400.5
Ethyl acetate	356.5
Butanol	63.0
Methanol	529.5
Water	–

3.2. Total flavonoids content

Total flavonoid content of all leaves crude extracts of neem was determined by aluminum chloride method based on quercetin standard curve (Fig. 3) (Hossain et al., 2013). The highest amount of total flavonoids was in methanol (529.5 mg/100 g dry powder samples) and the lowest was in butanol (63.0 mg//100 g dry powder samples) and followed sequentially methanol (529.5 mg/100 g), hexane (491.0 mg/100 g), chloroform (400.5 mg/100 g), ethyl acetate (356.5 mg/100 g), and butanol (63.0 mg/100 g) (Table 2).

3.3. Antimicrobial activity

The antimicrobial activity was determined by the disc diffusion method against four different bacterial strains (Hossain et al., 2014a,b). Six leaves crude extracts at different concentration were used to determine their antimicrobial activity by disc diffusion method; a standard amoxicillin antibiotic was used in this study. The result obtained from this study is presented in Table 3. Most of the crude extracts from the stems of neem did not show any potential activity against the employed Gram (+ and −) bacterial strains. The ethyl acetate crude extract at concentrations 2 and 1 mg/ml showed moderate activity against all employed food borne pathogenic bacterial strains. However, the ethyl acetate crude extract did not show any activity against all employed food borne pathogenic bacterial strains at the concentrations of 0.5 and 0.25 mg/ml. Hexane leaves crude extract showed moderate activity against *E. coli*, *S. aureus* and *P. aeruginosa* at the concentration 0.25 mg/ml but other concentrations did not show any activity. Chloroform also showed moderate activity against all employed bacterial strains at the concentration 0.25 mg/ml. Butanol showed slightly high activity among the six crude extracts obtained from the leaves of neem. Methanol crude extracts at concentration 2 mg/ml showed activity of 7–11 mm against *E. coli*, *S. aureus* and *P. aeruginosa*. However, *Vulgaris* bacterial strain did not show any response to most of the leaves crude extracts at all concentrations (Table 3).

4. Discussion

Neem was chosen for this study due to their medicinal values as well as being indigenous plant. It belongs to the family Meliaceae. There is lack of scientific information on the medicinal importance of this plant. Solvent of different polarities was used on neem leaves crude extracts for the determination of total phenolics and flavonoids contents. The results of total phenolics content of six leaves crude extract are presented in Table 1. The total phenolics content of the crude extracts from the leaves was expressed as gallic acid equivalents (GA) per

Table 3 – Antimicrobial activity of different leaves crude extracts.

Extracts	Conc.	*E. coli* (mm)	*S. aureus* (mm)	*P. aeruginosa* (mm)	*P. vulgaris* (mm)
Hexane	2 mg/ml	nd	nd	nd	nd
	1 mg/ml	nd	nd	nd	nd
	0.5 mg/ml	nd	nd	nd	nd
	0.25 mg/ml	9 ± 0.35	10 ± 0.55	6 ± 0.55	nd
	Standard	9 ± 0.13	9 ± 0.29	nd	nd
Ethyl acetate	2 mg/ml	7 ± 0.21	7 ± 0.37	12 ± 0.15	8 ± 0.29
	1 mg/ml	6 ± 0.43	9 ± 0.17	10 ± 0.14	9 ± 0.32
	0.5 mg/ml	nd	nd	nd	nd
	0.25 mg/ml	nd	nd	nd	nd
	Standard	nd	10 ± 0.22	7 ± 0.11	nd
Chloroform	2 mg/ml	nd	nd	nd	nd
	1 mg/ml	nd	nd	nd	nd
	0.5 mg/ml	nd	nd	nd	nd
	0.25 mg/ml	8 ± 0.22	12 ± 0.26	6 ± 0.39	12 ± 0.45
	Standard	nd	9 ± 0.25	nd	nd
Butanol	2 mg/ml	7 ± 0.24	7 ± 0.29	7 ± 0.18	nd
	1 mg/ml	nd	nd	nd	nd
	0.5 mg/ml	nd	nd	nd	nd
	0.25 mg/ml	nd	nd	nd	nd
	Standard	9 ± 0.12	8 ± 0.56	6 ± 0.72	nd
Methanol	2 mg/ml	10 ± 0.65	11 ± 0.27	7 ± 0.17	nd
	1 mg/ml	nd	nd	nd	nd
	0.5 mg/ml	nd	nd	nd	nd
	0.25 mg/ml	nd	nd	nd	nd
	Standard	nd	9 ± 0.30	nd	nd
Water extract	2 mg/ml	nd	nd	6 ± 0.15	nd
	1 mg/ml	nd	4 ± 0.10	nd	nd
	0.5 mg/ml	nd	nd	nd	nd
	0.25 mg/ml	nd	nd	nd	nd
	Standard	nd	8 ± 0.23	nd	nd

nd = Not detected.

gram of dry extract, which ranged between 20.80 and 107.29 mg GA/g. From Table 1, the highest amount of total phenolics was obtained in butanol crude extract and the lowest was from chloroform crude extract. The order of total phenolics was butanol > ethyl acetate > water > methanol > hexane > chloroform. Gallic acid was used as a standard calibration curve (Table 1 and Fig. 1).

Five leaves crude extracts were used for the determination of total amount of flavonoids content shown in Table 2. Total flavonoids content of different leaves crude extracts from neem was determined using UV–visible spectrophotometric method by aluminum chloride method (Hossain et al., 2013). The highest amount of total flavonoids was in methanol (529.5 mg/100 g dry powder samples) and the lowest was in butanol (63.0 mg/100 g dry powder samples); the sequentially order was methanol (529.5 mg/100 g), hexane (491.0 mg/100 g), chloroform (400.5 mg/100 g), ethyl acetate (356.5 mg/100 g), and butanol (63.0 mg/100 g) among the five leaves crude extracts. The entire plant kingdom contains flavonoids which are important components of human and animal diet. The different biological activities depend on the plant secondary metabolites and the regular consumption of them by human being may have serious consequences for health, both positive and negative (Mossini et al., 2009).

The antimicrobial result obtained from this study was presented in Table 3. Most of the crude extracts from leaves of neem did not show any significant activity against the employed Gram (+ and −) bacterial strains. The ethyl acetate crude extract at concentrations 2 and 1 mg/ml showed moderate activity against all employed food borne pathogenic bacterial strains. However, ethyl acetate crude extract did not show any activity against all employed food borne pathogenic bacterial strains at the concentrations of 0.5 and 0.25 mg/ml. Hexane leaves crude extract showed moderate activity against *E. coli*, *S. aureus* and *P. aeruginosa* at the concentration 0.25 mg/ml but other concentrations did not show any activity. Chloroform also showed moderate activity against all employed bacterial strains at the concentration 0.25 mg/ml. Butanol showed slightly high activity among the six crude extracts obtained from the leaves of neem. Methanol crude extracts at concentration 2 mg/ml gave activity in the range of 7–11 mm against *E. coli*, *S. aureus* and *P. aeruginosa*. However, *Vulgaris* bacterial strain did not show for most of the leaves crude extracts at all concentrations. The total phenolics, flavonoids and antimicrobial activities of different leaves crude extracts from the neem were demonstrated by many previous studies (Mossini et al., 2009; Chaisawangwong and Gritsanapan, 2009; Girish and Shankara Bhat, 2013; Stobiecki and Kachlicki, 2011; Halijah et al., 2011).

5. Conclusion

From the study, it may be concluded that leaves crude extracts of neem are a good source of phytochemicals. Different extracts showed significant total phenolics and flavonoids contents. Hence, further studies are needed to isolate pure compounds from the crude extract and to better understand the mechanism of such actions scientifically.

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