Pacific Science Review A: Natural Science and Engineering 18 (2016) 128–131

HOSTED BY

Contents lists available at ScienceDirect

Pacific Science Review A: Natural Science and Engineering

journal homepage: www.journals.elsevier.com/pacific-science-review-a-natural-science-and-engineering/

Biological activities of different neem leaf crude extracts used locally in Ayurvedic medicine

Zainab Saif Saleh Al-Hashemi, Mohammad Amzad Hossain*

School of Pharmacy, College of Pharmacy and Nursing, University of Nizwa, P.O. Box 33, 616, Nizwa, Oman

ARTICLE INFO

Article history:
Received 31 August 2016
Received in revised form
29 September 2016
Accepted 29 September 2016
Available online 19 November 2016

Keywords:
Neem
Biochemical screening
Total phenol content
Antioxidant activity
UV–visible spectroscopy
Malaria

ABSTRACT

Neem (*Azadirachta indica*) is widely used in the Ayurvedic medicine system for treating malaria and fever. The present study was undertaken to prepare crude extracts of neem leaves with different polarity organic solvents by using a maceration method and assessing phytochemical screening, the total phenol content and antioxidant activity by the spectroscopic method. The leaf samples collected from the Bahla, Ad Dakliyah region were used for extraction by a maceration method using different organic solvents with increasing polarities. Biochemical screening was determined by established methods. The total phenol content and free radical scavenging activity were assessed by conventional Folin–Ciocalteu reagent (FCR) and α, α-diphenyl-β-picrylhydrazyl (DPPH) methods. Biochemical screening of the crude extracts of neem leaves revealed positive results of flavonoids, saponins, steroids, alkaloids, amino acid and tannins. However, triterpenoid and anthraquinone were not detected in the crude extracts of neem leaves. The total phenol content of crude extracts from the leaves of the plant selected to be the gallic acid equivalent was found to be the highest in the ethyl acetate extract containing phenol compounds (3.58 g/100 g of dry powder) and the lowest in the water extract (0.42 g/100 g of dry powder). The highest antioxidant activity was found in the butanol extract and the lowest was in the hexane extract of the plant selected to be equivalent to DPPH. The crude extracts of neem showed significant antioxidant activity; thus, these extracts could be used as natural antioxidants for the preparation of medicines to treat different diseases.

1. Introduction

Neem (*Azadirachta indica*) is an evergreen tree that belongs to the Meliaceae family and is found throughout world [1]. Its English name is neem, and its Arabic name is Al Shurisha. Neem is a large tree that is approximately 25 meters in height with a semi-straight trunk. It is a flowering plant and normally starts fruiting after 3–5 years. The tree becomes productive within 10 years [2]. The bark of this tree is grey and rough. The leaves are up to 30 centimetres long. Each leaf has 10–12 serrated leaf lets that are 7 centimetres long by 2.5 centimetres wide. The neem tree grows well in minimum rainfall countries [3]. All parts of the selected plant are used as medicine for the treatment of many diseases and illnesses. Traditionally, the leaves and their paste are used for curing allergic skin reactions and antivirally treating smallpox and chicken pox [4]. Most urban Nepalese, Indian and Bangladeshi use neem twigs to clean their teeth. The juice from the leaves is used as a tonic to increase appetite and to remove intestinal worms [5]. It is also used for its hypoglycaemic, hypolipidemic, hepatoprotective and hypotensive activities and to control fever [6]. Therapeutically, the leaf extract is used for its antimicrobial activity against dental pathogens [7]. In addition, in the Ayurvedic medicine system, the selected plant is used to treat malarial fever [7,8]. Neem oil is very useful in the preparation of mosquito-repellent tablets and is now available in north-east India [5–7]. There are also several medicinal uses of neem, and its formulated products include treatments for cancer, skin diseases, digestive disorders and AIDS [9]. In Oman, it is used traditionally for the treatment of fever and diabetes. Several active chemical compounds are present in the selected plant, including glycosides, dihydrochalcone, coumarin, tannins, zadirachtin, nimbin, nimbidine, diterpenoids, triterpenoids, proteins,

* Corresponding author. Fax: +968 25446236.
E-mail address: hossainabi@gmail.com (M.A. Hossain).

Peer review under responsibility of Far Eastern Federal University, Kangnam University, Dalian University of Technology, Kokushikan University.

http://dx.doi.org/10.1016/j.psra.2016.09.013

carbohydrates, sulphurous compounds, polyphenolics, among others [10,11]. Of these, the most commonly active compounds found in neem are azadirachtin, nimbin and nimbidine [10]. The most active chemical compounds are slightly hydrophilic in nature; however, they are freely lipophilic and more soluble in organic solvents, such as water, alcohol, ketones and esters [12]. A literature survey revealed no publications on phytochemical screening, the total phenol content or antioxidant activity of Omani neem species. Therefore, this is the first report on phytochemical screening, total phenols and antioxidant activity of different crude extracts of the selected plant collected from Oman. The present study was conducted to evaluate the biochemical characteristics as well as the total phenol content and antioxidant activity of the selected plant crude extracts by FCR and DPPH methods.

2. Materials and methods

2.1. Materials

DPPH (2,2-diphenyl-1-pikryl-hydrazyl), butanol and ethyl acetate were obtained from Sigma–Aldrich Company, Germany. Acetone, chloroform and hexane were obtained from Daejung, Korea. Gallic acid was obtained from New Jersey, USA. Filter papers were obtained from Whatmann No 41, UK. Anhydrous sodium carbonate was obtained from industrial Estate, Mumbai, India. Methanol was obtained from Analar Normapur, France. The Folin–Ciocalteu reagent was obtained from Scharlau, Spain. UV spectroscopy (Shimadzu spectrophotometer, Model UV-1800, Japan) was used to measure the absorbance of the samples.

2.2. Plant sample

The neem leaf samples were collected from Bahla on February 7, 2014, at 8.30 am. The collected samples were packed in plastic bags and were transported to the Pharmacy Research Laboratory, University of Nizwa for further necessary steps.

2.3. Sample preparation

The leaf samples were washed with water to remove dust and foreign particles. The leaves were separated from the stems and were dried at 40–45 °C for 5 days. After drying, the leaves were ground into a powder by using a grinder. The leaf powder sample was kept in an amber coloured bottle.

2.4. Extraction procedure

The powdered sample (140 g) was extracted with a methanol solvent (260 ml) by using a maceration method for 3 days. After extraction, the sample was filtered by using a Bruckner funnel. The methanol solvent was evaporated by using a rotary evaporator under reduced pressure at 20 °C for 1 h. The crude extract became a semi solid mass (11.15 g; 7.96%). The methanol semi solid mass (0.53 g) was transferred into a plastic tube for antioxidant activity, total phenols and biochemical screening tests. The remaining methanol semi solid mass (10.62 g) was dissolved in distilled water (120 ml) and was shaken until the crude extract dissolved. The water solution was transferred into a separatory funnel and fractionation by 30 ml and 20 ml of hexane, chloroform, ethyl acetate and butanol [13]. After extraction, all fractions were kept inside the fume hood for evaporation of the mother solvents to give hexane (0.22 g; 2.07%), chloroform (1.28 g; 12.05%), ethyl acetate (0.24 g; 2.25%) and butanol crude extract (2.34 g; 21.84%). Finally, the remaining water fraction was evaporated to give a water crude extract (1.35 g; 12.71%).

2.5. Total phenol contents

Ten percent Folin–Ciocalteu reagent (FCR) and 6% sodium carbonate (Na_2CO_3) were prepared by adding water. Four milligrams of each crude extract of neem leaves, such as hexane, chloroform, ethyl acetate, butanol, methanol and water extracts, were taken separately in the test tube and dissolved in 4 ml of methanol. Two-hundred microliters of each sample was transferred into the other test tubes. One and a half millilitres of 10% FCR was added to it and was kept for 5 min in a dark place. One and a half millilitres of 6% sodium carbonate was added to each test tube and was shaken well, and all tubes were kept in the dark for 2 h. The absorbance was recorded by using a UV-visible spectrophotometer at a wavelength of 760 nm [14].

2.6. Antioxidant activity assay

Two milligrams of each crude extract of the selected neem leaves, such as hexane, chloroform, ethyl acetate, butanol, methanol and water crude extracts, were taken in a test tube and dissolved with 10 ml of methanol. Then, different concentrations, including 200, 100, 50, 25 and 12.5 μg/ml, were prepared by using a serial dilution technique. Two and a half millilitres of a 0.004% DPPH solution was added to all test tubes, shaken gently by hand and kept in a dark place for one and a half hours. The absorbance of all of the concentrations of crude samples was measured by using UV–visible spectroscopy at a wavelength of 517 nm. Finally, the antioxidant activity of all crude extract samples was calculated by using the following formula:

$$\%\ \text{inhibition} = A_{\text{standard}} - A_{\text{extract}}/A_{\text{standard}} * 100$$

2.7. Biochemical screening test

2.7.1. Test for alkaloids

The powdered leaf samples (1 g) were taken in a beaker, 3 ml of ammonia solution were added to it and the solution was kept for five minutes. Then, 10 ml of chloroform was added to the beaker and stirred. It was filtered to remove powdered samples. The chloroform solvent was evaporated to dryness by using a water bath; then, 2 ml of Mayer's reagent as added. A cream coloured precipitate was immediately produced, indicating the presence of alkaloids.

2.7.2. Test for steroids

The leaf powdered samples (1 g) were taken in a beaker, and 10 ml of chloroform was added to the beaker. The powdered samples were removed by filtering. Chloroform was evaporated to dryness by using a water bath. Then, 1 ml of acetic anhydride and 1 ml of sulphuric acid were added to the beaker. A green colour appeared, indicating the presence of steroids.

2.7.3. Test for tannins

The leaf powdered samples (1 g) were taken in a beaker, and ferric chloride (1 ml) was added to the beaker. A brownish black colour appeared, indicating the presence of tannins.

2.7.4. Test for flavonoids

One millilitre of each crude stock extract was taken in a test tube and a few drops of dilute sodium hydroxide were added. An intense yellow colour appeared in the test tube that became colourless with the addition of a few drops of dilute acid, indicating the presence of flavonoids.

2.7.5. Test for saponins

Each crude extract stock solution (1 ml) was taken in a test tube and was diluted with 20 ml of distilled water. Then, it was agitated

by hand for a few minutes. The formation of the foam layer showed the presence of saponins.

2.7.6. Test for amino acids

Each crude extract solution (1 ml) was taken in a test tube, and a few drops of Ninhydrin reagent were added. A purple colour appeared, indicating the presence of amino acids.

2.7.7. Test for anthraquinones

One millilitre of each crude stock extract solution was taken in a test tube and hydrolysed with a diluted concentrated sulfuric acid. It was extracted with benzene. A dilute ammonia solution was added to the benzene layer. The appearance of a rose pink coloration suggested a positive response for anthraquinones.

2.7.8. Test for triterpenoids

Five milligrams of each crude plant extract were dissolved in chloroform (5 ml). One millilitre of acetic anhydride and concentrated sulfuric acid were added to it. The formation of a reddish violet colour indicates the presence of triterpenoids.

3. Results and discussion

Plants and their products have been used extensively and safely for the treatment of medical problems [5]. Traditionally, medicinal plants play a vital role in developing countries for basic health needs [8]. However, herbal remedies have been used in developed countries since ancient times [7]. Because of their medicinal importance, plants and their products continue to be a rich source of therapeutic agents. For most of the drugs that are available in the world, the active ingredients are found in plant sources. Those active ingredients play a vital role in the treatment of diseases. The drug industry has used medicinal plants for manufacturing new drugs for the treatment of different diseases and illness. Phytochemical and biological studies have already been performed on a large number of plants by scientists all over the world. Therefore, our interest is in carrying out a screening of undetermined plants that are available in Oman to validate their use in folk medicine.

The neem leaf samples were collected locally and extracted by a maceration method with methanol. The semisolid masses were obtained from the leaf powder samples by methanol evaporation. The methanol crude extract was defatted with water. The defatted crude extract was extracted with hexane, chloroform, ethyl acetate and butanol solvent to give hexane, ethyl acetate, chloroform and butanol extracts, respectively (Table 1).

The crude extracts from neem were used to conduct different biochemical studies. Biochemical screening was performed according to well-established methods. The results showed that alkaloids, steroids, flavonoids, tannins, saponins and amino acid were present in all polarities of crude neem leaf extracts except anthraquinone and triterpenoids (Table 2). However, none of the crude extracts showed any colour change for the anthraquinone and triterpenoids test [15] (Table 2).

Table 1
Mass amounts of hexane, chloroform and ethyl acetate, butanol, methanol and water crude extracts from neem leaves.

Extract	Leaves (gm)
Hexane	0.22
Ethyl acetate	0.24
Chloroform	1.28
Butanol	2.34
Methanol	11.15
Water	2.34

Table 2
Phytochemical screening of hexane, chloroform, butanol, ethyl acetate, methanol and water crude extracts from neem leaves.

Phytochemicals	Inference					
	Hexane	Butanol	Ethyl acetate	Chloroform	Methanol	Water
Alkaloids	+	+	+	+	+	+
Steroids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Amino acid	+	+	+	+	+	+
Flavonoid	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Anthraquinone	–	–	–	–	–	–
Triterpenoids	–	–	–	–	–	–

+ = presence; – = absence.

The total phenol content of all crude extracts from neem leaves was determined by the Folin–Ciocalteu method as reported from the gallic acid equivalents (Table 3) [14]. Among the six crude extracts, the ethyl acetate crude extract contained the highest amount of phenol compounds (3.58 mg/100 g dry powder sample), and the lowest was in the water crude extract (0.42 mg/100 g dry powder sample) followed by the methanol extract (1.27 mg/100 g dry powder sample), butanol extract (1.00 mg/100 g dry powder sample), chloroform extract (0.96 mg/100 g dry powder sample) and hexane extract (0.90 mg/100 g dry powder sample) (Table 3 and Fig. 1).

In this study, the antioxidant activity of leaf crude extracts was investigated by *in vitro* models using the α, α-diphenyl-β-picrylhydrazyl (DPPH) method [16]. The mechanism of DPPH free radical scavenging activity is well known [17,18]. The highest antioxidant activity was found in the butanol extract and the lowest was in the hexane extract followed by butanol > methanol > chloroform > ethyl acetate > water > hexane crude extracts (Table 4 and Fig. 2).

Table 3
Total phenol content of different crude extracts from neem leaves by the maceration method.

Crude extracts	Total phenolic content (%, w/w g/100 g powder samples)
Hexane	0.90
Ethyl acetate	3.58
Chloroform	0.96
Butanol	1.00
Methanol	1.27
Water	0.42

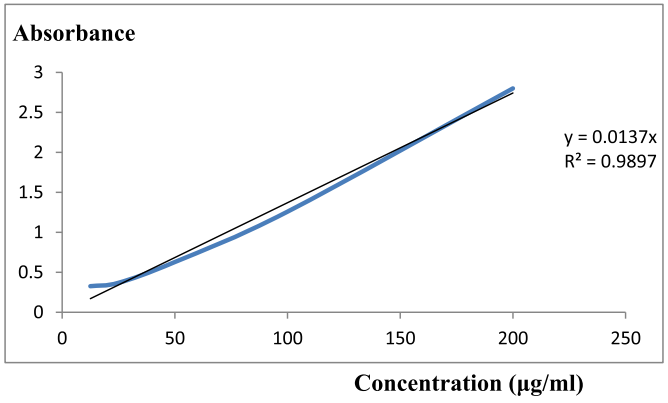

Fig. 1. Gallic acid standard curve.

Table 4
Antioxidant activity of hexane, butanol, ethyl acetate, chloroform, methanol and water crude extract from neem leaves.

Conc. (μg/ml)	Hexane	Butanol	% Ethyl acetate	Inhibition chloroform	Methanol	Water
12.5	30.91	48.60	35.01	35.01	31.28	31.01
25	34.54	62.29	35.38	35.10	33.52	35.19
50	41.34	76.16	37.80	43.02	44.13	39.01
100	43.95	88.08	47.49	56.70	56.70	42.46
200	56.52	90.97	59.96	66.39	81.66	56.79

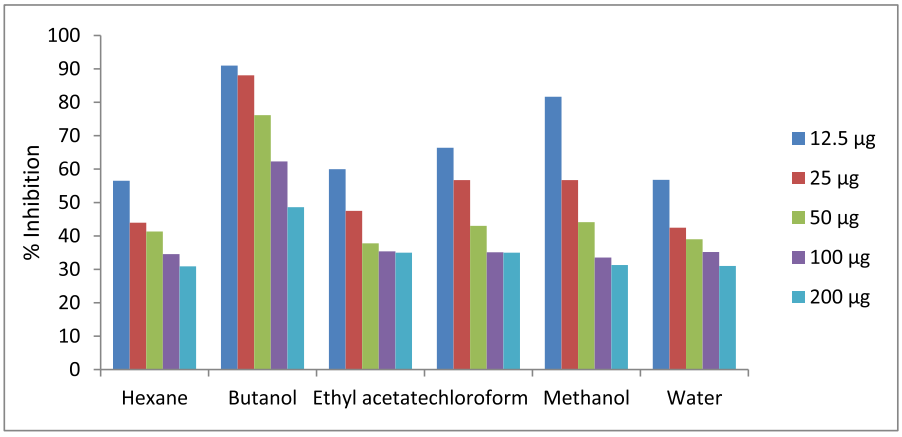

Fig. 2. Antioxidant capacity of the hexane, butanol, ethyl acetate, chloroform, methanol and water crude extract from neem leaves.

All of the crude extracts from neem showed positive tests for alkaloids, steroids, flavonoids, tannins, saponins and amino acids. The significant antioxidant activity of neem crude extracts might be the result of the presence of the above-mentioned phytochemicals. The almost similar results and relationship between he antioxidant activity of plant crude extracts and biochemical screening have previously been reported [17,18].

4. Conclusion

In the present study, analysis of the free radical scavenging activity and total phenol content showed that mainly the butanol and methanol crude extracts of the selected plant are potent sources of natural antioxidants. Therefore, the selected crude extracts can be used as a natural antioxidant instead of a synthetic antioxidant. Further studies are designed for the isolation and identification of individual phenolic compounds; also, *in vivo* studies are needed to better understand their mechanism of action as an antioxidant.

Acknowledgements

The authors are grateful to Prof. Dr. Nafsiah Binti Shamsudin, Dean, College of Pharmacy and Nursing, University of Nizwa, Sultanate of Oman for her continuous encouragement. Thanks to the University of Nizwa, Nizwa, Sultanate of Oman for providing all chemicals and other expenses from their internal funds to carry out this graduation project.

References

[1] P. Sithisarn, W. Gritsanapan, Free radical scavenging activity and total flavonoid content of siames neem tree leaves aqueous extract from different locations, Mohidol Uni. J. Pharma. Sci. 32 (1–2) (2011) 31–35.
[2] S. Maragathavalli, S. Brindha, N.S. Kaviyarasi, B. Annadurai, S.K. Gangwar, Antimicrobial activity in leaf extract of neem, Inter. J. Sci. Nat. 3 (1) (2012) 110–113.
[3] M.B. Mohashine, M. Nishimura, S. Matsumura, T. Shimono, Antibacterial effect of the crude *azadirachta indica* neem barks extract on *Streptococcus Sobrinus*, Paed. Dent. J. 7 (4) (2009) 61–64.
[4] K.H. Khine, M.A. Mon, M.N. Ha, Some chemical analyses and determination of antioxidant property of neem leaves, Uni. Res. J. 4 (3) (2013) 1–9.
[5] A.G. Kumar, C.J. Wu, B.J. Kumar, D.C. Ha, Antioxidant activity and quantitative estimation of azadirachtin and nimbin in *Azadirachta indica* A. Juss grown in foothills of Nepal, Afr. J. Biot. 8 (13) (2009) 3084–3091.
[6] P. Chaturvedi, A. Bag, V. Rawat, S.N. Jyala, V. Satyavali, P.K. Jha, Antibacterial effects of *Azadirachta indica* leaf and bark extracts in clinical isolates of diabetic patients, Natl. J. Integr. Res. Med. 2 (1) (2011) 5–9.
[7] I. Khan, S.S. Rao, S. Darsipudi, S.G. Divya, H. Amaranad, Phytochemical studies and screening of leaves extracts of *Azadirachta indica* for its anti-microbial activity against dental pathogens, Arch. Appl. Sci. Res. 2 (2) (2010) 246–250.
[8] U.B. Gochukw, A. Anyaehie, Medicinal properties of leaves extract from Nigeria, Niger. J. Physiol. Sci. 24 (2) (2009) 157–159.
[9] V.K. Santhosh, V. Navartnam, Neem prehistory to contemporary medicinal uses to humankind, Asian Pac, J. Trop. Biomed. 3 (7) (2013) 505–519.
[10] N.K. Mondali, A. Mojumdar, S.K. Chatterje, A. Banerjee, J.K. Datfa, S. Gupta, Antifungal activities and chemical characterization of neem leaves extracts on the growth of some selected fungal species in vitro culture medium, J. Appl. Sci. Environ. 13 (1) (2009) 49–53.
[11] P.K. Sudhir, D. Mishra, P. Ghosh, Biological action and medicinal properties of various constituent of *Azadirachta indica*, Ann. Biol. Res. 1 (3) (2010) 24–34.
[12] S. Pankaj, T. Lokeshwar, B. Mukesh, B. Vishnu, Review on neem thousand problem one solution, Inter. Res. J. Pharm. 2 (12) (2011) 79–102.
[13] C. Bempah, A. Kwofie, J. Asomaning, Morphological studies of neem sees and physicochemical properties of its oil extracts collected in Accra metropolis of Ghana, Appl. Bot. J. 39 (2011) 4951–4953.
[14] S.P.A. Aziza, M.A. Hossain, Study on total phenolics and antioxidant activity of leaves crude extracts of *Annona squamosal* traditionally used for the treatment of cancerous tumours, Asian pac. J. Trop. Dis. 5 (1) (2015) S142–S144.
[15] I. Hashmat, H. Azad, A. Ahmed, Neem (*Azadirachta indica* A. Juss)-Nature's drugstore, Inter. Res. J. 1 (6) (2012) 76–79.
[16] A. Aslam, K. Rehman, M. Asghar, M. Sarwar, Antibacterial activity of various phytoconstituents of neem, Pak. J. Agri. Sci. 46 (3) (2009) 209–213.
[17] G. Pandey, K. Verma, M. Singh, Evaluation of phytochemical antibacterial and free radical scavenging properties of neem leaves, Inter. J. Pharm. Pharm. Sci. 6 (2014) 1484–1491.
[18] S. Gunasekaran, B. Anita, Analysis of phytochemical variability in neem formulation, Indian J. Nat. Prod. Resour. 1 (3) (2010) 291–295.