Innovare
Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 2, 2015

Original Article

CYTOTOXICITY AND ANTIMICROBIAL PROPERTIES OF NEEM (*AZADIRACHTA INDICA*) LEAF EXTRACTS

YUVANESWARAN KRISHNAN, NYET KUI WONG*

Biotechnology Program, Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia.
Email: nkwong@ums.edu.my

Received: 25 Oct 2014 Revised and Accepted: 18 Nov 2014

ABSTRACT

Objective: This study was conducted to evaluate the biological properties of Neem (*Azadirachtaindica*) leaves in terms of its cytotoxic and antimicrobial activities.

Methods: The antimicrobial activity of *A. indica* leaf extracts were evaluated against pathogenic bacteria such as *E. coli, B. subtilis, B. cereus, S. pneumonia, S. aureus* and *P. aeruginosa* by using agar disc diffusion method. The cytotoxic activity of the plant extracts was tested using Brine Shrimp Lethality Assay.

Results: Acetone extract exhibited stronger inhibition against gram negative bacteria (*E. coli* and *P. aeruginosa)* with MIC values of 10mg/ml and 25mg/ml respectively. In contrast, chloroform extract exhibited stronger inhibition against gram positive bacteria (*B. subtilis*, *B. cereus, S. pneumonia*, and *S. aureus)* with the MIC value of 10mg/ml for all bacteria. The cytotoxicity that was evaluated based on the LC_{50} values of the extracts. Results showed that acetone extract has higher cytotoxicity than chloroform extract with the LC_{50} values of 6.00µg/ml and 11.92µg/ml respectively.

Conclusion: The study demonstrated that both acetone and chloroform extracts of *A. indica* leaves have strong antimicrobial and cytotoxic activities.

Keywords*:* Azadirachtaindica, Acetone Extract (AE), Chloroform Extract (CE), cytotoxicity, Antimicrobial activity.

INTRODUCTION

Plants have formed the basis of traditional medicine systems to maintain human health for thousands of years and have been used as valuable sources of natural products. The medicinal plants are laden with numerous effective pharmacological agents that provide an alternative means of therapy to various infections caused by drug resistant bacteria or dreadful diseases like cancer and other physiological disorders [13].

Azadirachta indica commonly known as Neem, belongs to Meliaceaefamily and it is well known in India and its neighbour countries for more than 200 years as one of the most versatile medicinal plant that has a wide spectrum of biological activity [1]. The first indication that Neem was being used in medical treatment was about 4500 years ago during the high point of the Indian Harappa culture, one of the greatest civilisations of the ancient world [6]. In fact, neem (*A. indica*), is the most useful traditional medical plant in India. Every part of the tree has been used as a traditional medicine for household remedy against various human ailments, from antiquity [1]. Besides that, Neem has been extensively used in Ayurveda, Yunani and homoeopathic medicine and has become a cynosure of modern medicine.

Neem has shown to be an excellent wound healer. The antiseptic and healing properties of neem make it an excellent first aid for minor cuts and abrasions [6]. The plant has the ability to increase vascular permeability by increasing the blood flow and by helping the body to rapidly create collagen fibers to close wounds. Neem also plays a role in treating skin burns. Besides, neem has been reported to have antipyretic compounds that have traditionally been used to reduce fevers. Studies done by Sharma *et al.,* 2011, has demonstrated that the methanol extract from neem leaves exert an antipyretic effect in male rabbits [19].

Neem is one of the few known anti-viral agents. Neem interacts with the surface of cells to prevent infection by inhibiting the multiplication of the virus. Mild neem leaf teas have been demonstrated to combat the chickenpox *varicella-zoster*virus and boost the immune system. It was found that neem can keep shingles, which is caused by the same virus, as chickenpox, at the surface level if took internally during times of stress. It can inactivate the viruses, and preventing the virus from multiplying sufficiently to cause an outbreak [6].

Therefore, the significance of this study is to identify the cytotoxicity and antimicrobial activities of the acetone extract and chloroform extract from neem leaf and determine the potential of these extracts to be the candidates for cancer therapy and strong antibiotics against pathogenic bacteria in the future.

MATERIALS AND METHODS

Collection and preparation of Neem *(Azadirachta Indica*) leaves

The neem (*A. indica*) leaves were collected from neem trees at Universiti Malaysia Sabah (UMS). Next, the leaves were washed under running tab water for 5 minutes in order to remove the dust particles stuck on the leaf surface. The leaves were allowed to dry for 10 days at room temperature. The dried leaves were then blended using dry blender to obtain the powder form of the leaf for more efficient and effective organic extraction.

Extraction of secondary metabolites

The dried *A. indica* leaves were ground to powder form. 50 g of each sample was soaked with chloroform and acetone in a closed 500 ml conical flasks and then kept at room temperature for 24 hours. The extraction process was repeated 2 times (extraction for 2 days). Then the extracts were filtered by using Whatman 1filter paper and concentrated at reduced pressure using a rotary evaporator at 40°C. The dried extracts were kept in the refrigerator at 4 °C for further use [2].

Antibacterial assay

The agar disc-diffusion assay was performed using the concentration of 100 mg/ml to evaluate antibacterial activity of acetone and chloroform extracts of neem leaf. Briefly, 100 ml of nutrient broth was inoculated with each test organism and incubated at 37°C for overnight. The 10 ml of nutrient agar was poured on the sterile Petri plates and were allowed to set and

harden for few minutes. 100 µl of nutrient broth culture was poured on each agar Petri plates and were spread by using sterilized glass spreader. The antibiotic Ampicillin drug was used as the control. The Petri plates were sealed and incubated at 37^{0}C for overnight. The diameter of the zones of inhibition was measured in mm [9].

Minimum inhibitory concentration (MIC) determination

Extracts that showed positive results in the disc diffusion assay against test pathogens were further evaluated for minimum inhibitory concentration (MIC) using six different concentrations (5, 10, 20, 40, 80, 100 µl/ml) and disc diffusion method was used. The lowest concentration that inhibits the growth of bacteria was noted and considered as the MIC value for each of the bacteria strain [12, 23].

Brine shrimp cytotoxicity test

Little amount of cysts was put into artificial marine water (1.2-3.0 % NaCl). The water was kept in mild motion by aeration with mild air flow, which gives enough air to the organisms. The water was kept at 27-29^{0}C for 24 hours so that hatching could take place in the presence of a light source. The toxicity of the extracts of neem leaf was tested at various concentrations such as 10, 100, and 1000 µg/mL in seawater containing 2% DMSO (v/v). Three replications were used for each concentration. Ten nauplii were used in each test. 1 % mercury chloride was used as positive control. DMSO and distilled water was used as negative controls. After 24 hours, survivors were counted using a dissection microscope and the percentage of the Lethality (%) was calculated using the following formula:

% Lethality of Brine Shrimps

$$= \left[\frac{\text{Total no. of Brine Shrimps} - (\text{No. of Brine Shrimps Survived})}{\text{Total no. of Brine Shrimps}}\right] \text{x } 100$$

The LC_{50} values of each sample were calculated from the graph of 1% lethality against concentration and compared with the control.

RESULTS

Extraction of secondary metabolites

Acetone extract has the higher net yield with 6.94 g (5.34 % recovery), and chloroform extract has the net yield of 4.60 g (3.54 % recovery). Since, acetone has a higher polarity index as compared to chloroform, acetone could extract more polar compounds [20] from neem *(A. indica)* leaves and chloroform extracted more non polar compounds. This result indicates that, neem *(A. indica)* leaves contain more polar compounds than non-polar compounds.

Antimicrobial assay and MIC test

Acetone extract (AE) showed stronger inhibition against both *E. coli* and *P. aerugenosa* which are gram negative bacteria with bigger inhibition zones for all the concentrations used from 10 mg/ml to 100 mg/ml when compared to the inhibition zones formed by chloroform extract (CE) for the similar bacteria (Table 1 and Table 2). On the other hand, CE showed stronger inhibition against *B. subtilis, B. cereus, S pneumonia* and *S. aureus* which are gram positive bacteria with bigger inhibition zones for all the concentrations used from 10mg/ml to 100 mg/ml. As showed in Table 3, the MIC of acetone extract for *E. coli, B. subtilis, B. cereus* and *S. pneumonia* is 10 mg/ml whereas, the MIC for *P. aerugenosa* and *S. aureus* is 25mg/ml. On the other hand, MIC of chloroform extract against all the bacteria is 10 mg/ml (Table 3).

Brine shrimp lethality assay

The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity, of which in most cases correlate reasonably well with cytotoxic and anti-tumor properties [7].

Table 1: Size of inhibition zone (mm) formed by different concentrations (mg/ml) of acetone extract (AE) of neem (*a. indica*) leaf against various bacteria

Concentration mg/ml	Size of inhibition zone (mm)					
	E. coli	***B. subtilis***	***B. cereus***	***S. pneumoniae***	***P. aerugenosa***	***S. aureus***
10	10.00±0.00	8.67±0.58	9.33±0.58	8.67±0.58	0.00±0.00	0.00±0.00
25	11.00±0.00	10.00±0.00	11.00±0.00	9.33±0.58	11.67±0.58	10.33±0.58
50	11.70±0.58	11.00±0.00	11.33±0.58	10.67±0.58	13.33±0.58	11.00±0.00
75	13.00±0.00	11.33±0.58	11.67±0.58	11.33±0.58	14.67±0.58	12.00±0.00
100	13.33±0.58	12.67±0.58	12.67±0.58	12.00±1.00	16.00±0.00	12.67±0.58
Positive Control	17.67±0.58	17.67±0.58	19.67±1.15	19.33±0.58	18.00±1.00	15.33±0.58
Negative control	0.00± 0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 2: Size of inhibition zone (mm) formed by different concentrations (mg/ml) of chloroform extract (CE) from neem (A. indica) leaf against various Bacteria

Concentration mg/ml	Size of Inhibition Zone (mm)					
	E. coli	***B. subtilis***	***B. cereus***	***S. pneumoniae***	***P. aerugenosa***	***S. aureus***
10	8.67 ± 0.58	8.67 ± 0.58	8.33 ± 0.58	9.00 ± 0.00	8.00 ± 0.00	8.33 ± 0.58
25	9.67 ± 0.58	10.00 ± 1.15	10.00 ± 0.00	10.67 ± 0.58	9.33 ± 0.58	10.33 ± 0.58
50	11.00 ± 0.00	11.33 ± 0.58	10.67 ± 0.58	11.67 ± 0.58	12.00 ± 1.00	11.67 ± 0.58
75	12.00 ± 0.00	12.00 ± 1.15	11.67 ± 0.58	13.00 ± 0.00	13.33 ± 0.58	13.67 ± 0.58
100	13.00 ± 0.00	13.33 ± 0.58	13.00 ± 0.00	14.00 ± 0.00	14.33 ± 0.58	15.33 ± 0.58
Positive Control	19.00 ± 2.00	19.33 ± 0.58	19.00 ± 2.00	19.00 ± 1.00	15.33 ± 1.15	17.33 ± 0.58
Negative control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 3: MIC (mg/ml) of acetone extract and chloroform extract of Neem (*A. indica*) leaf

Sample Extract	Minimum Inhibitory Concentration (MIC) for Different Bacteria (mg/ml)					
	E. coli	***B. subtilis***	***B. cereus***	***S. pneumoniae***	***P. aerugenosa***	***S. aureus***
Acetone Extract	10	10	10	10	25	25
Chloroform Extract	10	10	10	10	10	10

Note: At 5mg/ml no inhibition was observed. Thus, 10 mg/ml as MIC is valid

Artemiasalina was used due to the fact that its response to the bioactive agents similar to that of mammalians. When the LC_{50} value, which is the lethal concentration that can kill 50% of the tested shrimps, is less than 250 µg/ml, the cytotoxic activity of the

extract is considered significant and had the potential for further investigation [3,15]. On the other hand, when the LC_{50} value of an extract is more than 1000µg/ml, it is considered non-toxic. Both the acetone extract (AE) and chloroform extract (CE) from neem (*A. indica*) leaves showed high cytotoxicity against brine shrimp with the LC_{50} values 6.00 µg/ml and 11.92µg/ml respectively. Whereas, the positive control which was mercury chloride showed a LC_{50} value of 0.57µg/ml. The high lethality of the brine shrimps caused by AE and CE extracts of neem leaves is and indicative of the presence of potent cytotoxic components. These cytotoxic compounds from neem leave extracts have the potential for further purification for anticancer study. The graphical representation is presented in Fig 1. and Fig 2.

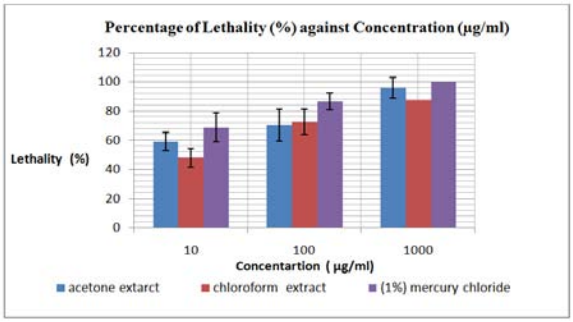

Fig. 1: Comparison between the percentages of lethality (%) of brine shrimps when tested with neem leaf extracts against various concentrations (10, 100 and 1000) µg/ml of acetone extract (AE), chloroform extract (CE), and mercury chloride as positive control

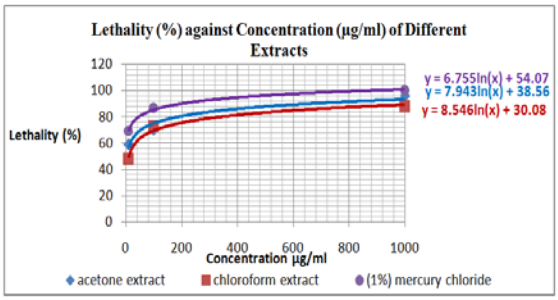

Fig. 2: Percentage of lethality (%) of brine shrimps against different concentration (µg/ml) of Neem (A. indica) Leaf extracts

DISCUSSION

The lower the MIC, the higher the antimicrobial activity of the extracts and therefore the more effective the extract is. Acetone extract (AE) showed stronger inhibition against the gram negative bacteria while Chloroform Extract (CE) showed stronger inhibition against the gram positive bacteria because different compounds in the AE and CE may interact differently with the bacteria in order to inhibit the growth. This is supported by the study done by Vinoth*et al* (2012), as the phytochemical analysis and antibacterial activity of *A. indica* showed that AE of neem leaf contains reducing sugar, saponin and glycosides [22]. On the other hand, the phytochemical analysis of CE of neem leaf showed the presence of glycosides, fatty acids and triterpenes [4]. From the two findings, it can be predicted that, saponin and reducing sugar from AE may play a vital role in gram negative bacterial growth inhibition, whilst, triterpenes and fatty acids from CE may play a vital role in the inhibition of gram positive bacterial growth.

The high lethality of the brine shrimps caused by AE and CE of neem leaf is and indicative of the presence of potent cytotoxic components. Azadirachtinthat is derived from the neem tree (*A. indica*) is both a toxicant and anti feedent which had an effect on brine shrimp lethality. PinmentelMontanher*et al* (2001) reported that the brine shrimp bioassay for general screening of Brazilian medical plants revealed that oleanolic acid which is a triterpene and coumarin was identified to have its cytotoxic effect on brine shrimps [16]. The partitioned ethanol crude extract of *Acacia Senegal* stem bark was found that the chloroform, n –hexane and methanol fractions recorded very low LC_{50} values of 27.21µg/ml, 6.77µg/ml and 27.38µg/ml. It was reported to be the presence of alkaloids, steroids, tannins and reducing sugars in common, while the n-hexane fraction with the lowest LC_{50} value had an additional different compound, saponin. Therefore this result indicates that, saponin can be a very potent cytotoxic compound [11].

CONCLUSION

A. indica leaf extracts has been proven to contain many medical values such as strong cytotoxicity and effective antimicrobial activities. The acetone extract and chloroform extract have good potential for cancer therapy as both the extracts have strong cytotoxicity activities. Therefore, further studies are necessary for the chemical characterization of the active compounds and more comprehensive biological assays of *A. indica* leaf extracts.

ACKNOWLEDGEMENT

The author is thankful to Dr. Ivy Wong NyetKui, Hong Chia Yean and GothaiSivapragasm for the guidance and support along the entire research.

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Ahmed MF, Rao AS. Simultaneous determination of phenolic compounds in *meliaazedarach. linn* leaves by high performance liquid chromatography. Indian J Appl Res 2013;3(11):2249-555X.
2. Anokuwuru CP, Anyasor GN, Ajibaye O, Fakoya O, Okebugwu P. Effect of extraction solvents on phenolic, flavonoid and antioxidant activities of three nigerian medicinal plants. J Nat Sci 2011;9(7):53-61.
3. Khor ES, Wong NK. Potential antioxidant and cytotoxic properties of secondary metabolite extracts from *carica papaya* fruits and seed. Int J Pharm Pharm Sci 2014;6(7):220-4.
4. Gayathri N, Rajani KS. *In vitro* antioxidative activity of *Azadirachtaindica*and *Meliaazedarach* Leaves by DPPH scavenging assay. J Nat Sci 2010;8:(4):22-8.
5. Irshad S, Butt M, Younus H. *In-Vitro* antibacterial activity of two medicinal plants neem *(azadirachtaindica*) and peppermint. Int J Pharm 2011;1(1):9-14.
6. John C. Neem the Ultimate Herb. Lotus Press: United States of America; 2009.
7. Krishnaraju AV, Rao TVN, Sundararaju D, Vanisree M, Tsay HS, Subbaraju GV. Assessment of bioactivity of indian medicinal plants using brine shrimp (*artemiasalina*) lethality assay. Int J Appl Sci Eng 2005;3(2):125-34.
8. Mahlo SM, Chauke HR, McGaw LJ, Eloff JN. Antioxidant and antifungal activity of selected plant species used in traditional medicine. J Med Plant Res 2013;7(33):2444-50.
9. Mondali NK, Mojumdar RA, Chatterje SK, Banerjee A, Datta JK, Gupta S. Antifungal activities and chemical characterization of Neem leaf extracts on the growth of some selected fungal species *in vitro* culture medium. J Appl Sci Environ Manage 2009;13(1):49-53.
10. Motaal AB, Shaker S. Anticancer and antioxidant activities of standardized whole fruit, pulp, and peel extracts of egyptian pomegranate. Open Conf Proc J 2011;2:41-5.
11. Mudi SY, Muhammad A. Antimalaria activity of ethalic extracts of leaves of *Terminaliacatappa. L. Combretaceae* (Indian Almond). Bayero J Pure Appl Sci 2009;2(1):14-8.
12. Muhammad Aizuddin AB, Thomas AN, Wong NK. Seawater dependency of marine actinomycetes for antioxidant and antimicrobial properties. Int J Pharm Pharm Sci 2014;6(4):96-101.
13. Neha C, Dolly S, Painuli RM. Screning of bioprotective properties and phytochemical analysis of various extracts of *eclipta alba* whole plant. Int J Pharm Pharm Sci 2012;4(2):975-1491.

14. Okhale SE, Amando MO, Jegede IA, Egharevba HO, Muazzam IW, Kunle OF. Phytochemical and pharmacognostic investigation of antidiabetic *scopariadulcis linn scrophulariaceae* whole plant grown in nigeria. J Pharmacol 2010;2(6):7-16.
15. Patil SB, Magdum CS. Determination of LC50 values of extracts of *euphorbia hirta linn* and *euphorbia neriifolia linn* using brine shrimp lethality assay. Asian J Res Pharm Sci 2011;1(2):42-3.
16. Pimentel MAB, Pizzolatti MG, Costa BIM. An application of the brine shrimp bioassay for general screening of brazilian medicinal plants. Acta Farm Bonaerense 2002;21(3):175-8.
17. Rahman AS, Arslan I, Saha R, Talukder N, Khaleque S, Araalf H. Bioactivity guided cytotoxic activity of *Clitoria Ternatea*utilizing brine shrimp lethality bioassay. Bangladesh J Physiol Pharmacol 2006;22(1/2):18-21.
18. Rajkumar V, Gunjan G, Ashok kumar R. Isolation and bioactivity evalution of two metabolites from methanolic extract of *oroxylumindicum* stem bark. Asian Pac J Trop Biomed 2012;7-11.
19. Sharma P, Tomar L, Bachwani M, Bamsal V. Review of Neem *(Azadirachtaindica*): thousand problems one solution. Int Res J Pharm 2011;2(12):97-102.
20. Tomsone L, Kruma Z, Galoburda R. Comparison of different solvents and extraction methods for isolation of phenolic compounds from horseradish roots (*armoraciarusticana*). J Sci Technol 2012;6(4):1164-9.
21. Ullah R, Ibrar M, Shah S, Hameed I. Phytotoxic, cytotoxic and insecticidal activities of *Calendula arvensis L.* J Biotechnol Pharm Res 2012;3(6):104-11.
22. Vinoth B, Manivasagaperumal R, Rajaravindran M. Phytochemical analysis and antibacterial activity of *Azadirachtaindica* A Juss. Int J Res Plant Sci 2012;2(3):50-5.
23. Zaidan MRS, Noor RA, Badrul AR, Adlin A, Norazah A, Zakiah I. *In vitro* screening of five local medicinal plants for antibacterial activity using disc diffusion method. J Trop Biomed 2005;22(2):165-70.