In vitro Organogenesis and Antibacterial activity in leaves extracts of Neem (*Azadirachta indica* A. Juss.)

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Abstract

The present study deal with *in vitro* organogenesis and antibacterial activity in leaves extracts of *Azadirachta indica* A. juss. *In vitro* organogenesis of Neem was using the basic medium CB and various concentration naphthylacetic acid (NAA) and benzylaminopurine (BAP). The callus were observed the concentration medium on 10^{-7} M (NAA) and 10^{-5} M (BAP), 10^{-6} M (NAA) and 10^{-5} M (BAP), 10^{-5} M (NAA) and 10^{-7} M (BAP), 10^{-5} M (NAA) and 10^{-5} M (BAP), 10^{-5} M (NAA) and 10^{-4} M (BAP). Maximum root regeneration were observed the concentration medium on 10^{-6} M (NAA) and 10^{-7} M (BAP), 10^{-5} M (NAA) and 10^{-7} M (BAP), 10-5 M (NAA) and 10-6 M (BAP), 10-5 M (NAA) and 10-5 M (BAP). Leaves extracts were used maceration by methanol, ethanol and distilled water (D/W). The antibacterial effects were investigated by the agar-well diffusion method against three gram positive strains, two gram negative strains and one strain of yeast fungus, which were obtained from the Development Centre of Pharmaceutical and Food Technology (DCPFT). The methanol and ethanol extracts showed the maximum inhibition zone on *Staphylococcus aureus*. The D/W extracts showed the maximum inhibition zone on *Bacillus pumilus*.

Key words: organogenesis, pathogenic bacteria, agar-well diffusion method

Introduction

Medicinal plants are of great value in the treatment and cure of diseases. The future development of the pharmacognostic analysis of herbal drugs is largely dependent upon reliable methodologies for correct identification, standardization and quality assurance of herbal drugs. Describing herbal drugs in a systematic manner is based on multiple approaches of pharmacognostic, taxonomic and chemical analysis, including documentation of their biological and geographical source, cultivation, collection and processing, morphological, microscopic and chemical characters.

It has been used in Ayurvedic medicine for more than 4000 years due to its medicinal properties. Neem is called “arista” in Sanskrit a word that means “perfect, complete, inperishable”. The importance of the Neem tree has been recognised by the US National Academy of Science, which publish a report in 1992 entitled “Neem- a tree for solving global problems (Girish K. and Bhat S. Shankara, 2008 and Kausik Biswas *et al*., 2002).

The increase in demand for herbal medicines may lead to indiscriminant and unscientific collection, quality of the material. According to Handa (2004), the majority of medicinal plants used by the herbal drug industry and local communities come from wild collection. The raw material used by the drug industry and communities in large cities, towns and regions is generally procured through market channels and is sometimes found adulterated. The neem tree *Azadirachta indica* A. Juss. (Meliaceae) is a tropical evergreen related to mahogany. Native to east India and Myanmar, it grows in much of Southeast Asia and West Africa. The evergreen tree is large, reaching 12 to 18 m in height with a girth of up to 1.8 to 2.4 m. Its blossoms are small, white flowers with a very sweet. Its fruit is about ¾ of an inch (2 cm) long, with white kernels. The shiny dark green pinnately compound leaves are up to 30 cm long. Each leaf has 10-12 serrated leaflets that are 7 cm long by 2.5 cm wide (Muňoz - Valenzuela *et al*., 2007).

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Neem has been extensively studied for its pesticidal and medicinal properties in Asiatic countries. Despite almost every part of the tree having a bitter taste, parts such as leaves, bark, flower, fruit, seed and root have advantages in medical treatment and industrial products. Its leaves can be used as drug for diabetes, eczema and reduce fever. Barks of Neem can be used to make toothbrush. Neem roots has an ability to heal diseases and against insects (Liauw *et al*., 2008).

Neem has many important agrochemical and economic uses. Due to highly heterozygous nature, long reproductive cycle and poor seed yield, improvement of neem by conventional methods is very limited. In this respect, tissue culture can play an important role. In vitro regeneration of neem from various vegetative tissues was published (Chaturvedi *et al*., 2004a, and Rout, 2005). Recently, some studies have described microspores (Chaturvedi *et al*., 2003a) and endosperm tissue (Chaturvedi *et al*., 2003b) of adult tree origin.

The importance of the neem tree has been recognized by the US National Academy of Science, which published a report in 1992 entitled "Neem-a tree for solving global problems". The advancement of neem research has earlier been documented (Schmutterer, 1995 and Singh, 1996).

In this study, the herbal drug Neem (*Azadirachta indica* A. Juss.) was selected as a case study for *in vitro* organogenesis and antibacterial activity of neem leaves against human pathogenic bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albican* and *E. coli*.

Materials and Methods

1. Prepare a double concentrated CB medium without phytohormones (1.5 l)

The basic CB medium (mg/ l): CB medium (Petersen and Alfermann, 1988)

Macronutrients:		**Micronutrients:**	
KNO_3	2,500	H_3BO_3	3
$MgSO_4$. 7 H_2O	250	$ZnSO_4$. 7 H_2O	3
NaH_2PO_4. H_2O	172	$MnSO_4$. H_2O	1
$CaCl_2$. 2 H_2O	150	KI	0.75
$(NH_4)_2SO_4$	134	Na_2MoO_4. 2 H_2O	0.25
$FeSO_4$. 7 H_2O	25.6	$CuSO_4$. 5 H_2O	0.25
Na_2-EDTA	34.3	$CoCL_2$. 6 H_2O	0.25
		Thiamine dichloride	10
		Pyridoxine	1
		Nicotinic acid	1
		Myo-inositol	300 mg
		Sucrose	6 g

pH 5.8 adjusted with 0.5 N HCl

2. Prepare a stock solution of naphthylacetic acid (NAA): 20 mg/ 100 ml = 1.07 mM (NAA-1) and the following dilutions:
   1:10 = 2 mg/100 ml (NAA-2)
   1:100 = 0.2 mg/100 ml (NAA-3)
   1:1000 = 0.02 mg/100 ml (NAA-4)
   NAA 20 mg/100 ml: Dissolve 20 mg naphthylacetic acid in 1 ml 96% ethanol and fill up with bidistilled water to 100 ml.
3. Prepare a stock solution of benzylaminopurine (BAP): 20 mg/100 ml= 0. 89 mM (BAP-1) and the following dilution.
   1:10 = 2mg/100 ml (BAP-2)
   1:100 = 0.2 mg/100 ml (BAP-3)
   1:1000 = 0.02 mg/100 ml (BAP-4)
   BAP 20 mg/100 ml: Dissolve 20 mg benzylaminopurine in 1 ml 0.5 M HCl and fill up with bidistilled water to 100 ml.
4. Prepare 25 Erlenmeyer flasks with 0.8 g agar- agar per flask and number them 1-25.
   Add to the flasks BAP, NAA and bidistilled water (w) according to the following table:

BAP	**0**	**10^{-7}M**	**10^{-6}M**	**10^{-5}M**	**10^{-4}M**
NAA					
0	BAP (0 ml) NAA (0 ml) W (50 ml)	BAP-4 (11.2 ml) NAA (0 ml) W (38.8 ml)	BAP-3 (11.2 ml) NAA (0 ml) W (38.8 ml)	BAP-2 (11.2 ml) NAA (0 ml) W (38.8 ml)	BAP-1 (11.2 ml) NAA (0 ml) W (38.8 ml
10^{-7}M	BAP (0 ml) NAA–4 (9.3 ml) W (40.7 ml)	BAP-4 (11.2 ml) NAA–4 (9.3 ml) W (29.5 ml)	BAP-3 (11.2 ml) NAA–4 (9.3 ml) W (29.5 ml)	BAP-2 (11.2 ml) NAA–4 (9.3 ml) W (29.5 ml)	BAP-1 (11.2 ml) NAA–4 (9.3 ml) W (29.5 ml)
10^{-6}M	BAP (0 ml) NAA-3 (9.3 ml) W (40.7 ml)	BAP-4 (11.2 ml) NAA-3 (9.3 ml) W (29.5 ml)	BAP-3 (11.2 ml) NAA-3 (9.3 ml) W (29.5 ml)	BAP-2 (11.2 ml) NAA-3 (9.3 ml) W (29.5 ml)	BAP-1 (11.2 ml) NAA-3 (9.3 ml) W (29.5 ml)
10^{-5}M	BAP (0 ml) NAA-2 (9.3 ml) W (40.7 ml)	BAP-4 (11.2 ml) NAA-2 (9.3 ml) W (29.5 ml)	BAP-3 (11.2 ml) NAA-2 (9.3 ml) W (29.5 ml)	BAP-2 (11.2 ml) NAA-2 (9.3 ml) W (29.5 ml)	BAP-1 (11.2 ml) NAA-2 (9.3 ml) W (29.5 ml)
10^{-4}M	BAP (0 ml) NAA-1 (9.3 ml) W (40.7 ml)	BAP-4 (11.2 ml) NAA-1 (9.3 ml) W (29.5 ml)	BAP-3 (11.2 ml) NAA-1 (9.3 ml) W (29.5 ml)	BAP-2 (11.2 ml) NAA-1 (9.3 ml) W (29.5 ml)	BAP-1 (11.2 ml) NAA-1 (9.3 ml) W (29.5 ml)

a) Add 50 ml of the double concentrated CB medium to each Erlenmeyer flask.
b) Autoclave as normal, shake the flasks to fully dissolve and distribute the agar.
c) Pour the medium into small Petri dishes and let them get hard and cool.
d) Place 4 about 0.5 cm long pieces of seedling hypocotyls to one Petri dish of each medium.
e) Close the Petri dish with Nescofilm and place the Petri dishes in the light.
f) Note observation of callus, shoot or root formation regularly.

Antibacterial tests

1. Collection of plants

The neem leaves were collected from Sagaing University Campus. The leaves were cleaned, dried at room temperature and ground to coarse powder for antibacterial tests.

2. Leaves extracts

Coarsely powder leaves of neem 50 g using maceration with 100 ml methanol, ethanol and D/W allowed one week respectively. Then filtered through Whatman No. 1 filter paper using funnel and the residue was compressed to ensure recovery of the filtrate.

3. Human Pathogenic Bacteria

Bacteria obtained from the Development Centre of Pharmaceutical and Food Technology (DCPFT). Three gram positive strains (*Bacillus subtilis*, *Staphylococcus aureus* and *Bacillus pumilus*), two gram negative strains of bacteria (*Pseudomonas aeruginosa* and *E. coli*) and one strain of yeast fungus (*Candida albican*).

4. Preparation of plates for test antibacterial activity

The antimicrobial activity tests were performed by agar-well diffusion method. Nutrient agar was prepared according to method described by Cruickshank, 1975. Nutrient agar was boiled and 20 - 25 ml of the medium was poured into a test tube and plugged with cotton wool and autoclaved at 121Ċ for 15 minutes. Then the tube was cooled down to 30-35Ċ and poured into sterilized petridishes and 0.2 ml of test organisms were also added into dishes. After that about 0.2 ml of sample was introduced into agar-well and incubated at 37Ċ for 24 hours. The inhibition zone appeared around the agar-well, indicating the presence of antimicrobial activity. The extent of antimicrobial activity was measured from the diameter zone of inhibition which resultant clear zones around the disc were measured in mm using a standard scale shown in Table (1).

Results and Discussion

In the present work, *in vitro* organogenesis of Neem were using the basic medium (CB), various concentration of stock solution of naphthylacetic acid (NAA) and benzylaminopurine (BAP). After 6 weeks, the callus inductions were observed the concentration medium on 10^{-7} M (NAA) and 10^{-5} M (BAP), 10^{-6} M (NAA) and 10^{-5} M (BAP), 10^{-5} M (NAA) and 10^{-7} M (BAP), 10^{-5} M (NAA) and 10^{-5} M (BAP), 10^{-5} M (NAA) and 10^{-4} M (BAP). Maximum root regeneration was observed the concentration medium on 10^{-6} M (NAA) and 10^{-7} M (BAP), 10^{-5} M (NAA) and 10^{-7} M (BAP), 10^{-5} M (NAA) and 10^{-6} M (BAP), 10^{-5} M (NAA) and 10^{-5} M (BAP). The shoot induction was observed on 10^{-5} M (NAA) and 10^{-6} M (BAP).

This medium supported direct rooting at the base of the shoot without an intervening callus phase. The present study, the presence of NAA in combination with BAP in the CB medium promoted callus induction from explants. Subsequently, these calli from proliferation medium were subcultured on regeneration medium to achieve organogenesis.

Figure (1) Habit of Neem

Figure (2) Seeds of Neem

Figure (3) Young plant of Neem

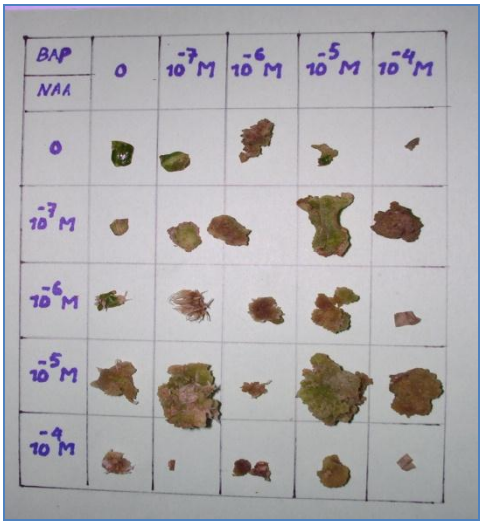

Figure (4) Organogenesis of Neem on various concentration media No. 1-25 (after 6 weeks)

The methanol extracts of Neem showed inhibition zone on *Bacillus subtilis* (20 mm), *Staphylococcus aureus* (27 mm), *Pseudomonas aeruginosa* (20 mm), *Bacillus pumilus* (22 mm), *Candida albican* (15 mm) and *E. coli* (25 mm). The ethanol extracts of Neem showed inhibition zone on *Bacillus subtilis* (16 mm), *Staphylococcus aureus* (28 mm), *Pseudomonas aeruginosa* (20 mm), *Bacillus pumilus* (20 mm), *Candida albican* (17mm) and *E. coli* (25 mm). The D/W extracts of Neem showed inhibition zone on *Bacillus subtilis* (12 mm), *Staphylococcus aureus* (14 mm), *Pseudomonas aeruginosa* (15 mm), *Bacillus pumilus* (17 mm). The inhibition zones were not show on *Candida albican* and *E. coli*. The extent of antimicrobial activity was measured from the diameter zone of inhibition which resultant clear zones around the disc were measured in (mm) using a standard scale shown in Table (1) figure (5).

Table (1) antibacterial activity of Neem (*Azadirachta indica* A. Juss.) leaves extracts from methanol, ethanol and distilled water (D/W)

Date	Sample	Solvent	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*B. pumilus*	*C. albican*	*E.coli*
3.6.2015	Neem	MeOH	20 mm	27 mm	20 mm	22 mm	15 mm	25 mm
		EtOH	16 mm	28 mm	20 mm	20 mm	17 mm	25 mm
		D/W	12 mm	14 mm	15 mm	17 mm	-	-
	Control	MeOH	-	-	-	-	-	-
		EtOH	-	-	-	-	-	-
		D/W	-	-	-	-	-	-

Agar well -10 mm
10 mm~14 mm (+)
15 mm~19 mm (+ +)
20 mm above (+ + +)
(-) Not active

Human pathogenic bacteria:
(1) *Bacillus subtilis* (N.C.T.C-8236)
(2) *Staphylococcus aureus* (N.C.P.C.-6371)
(3) *Pseudomonas aeruginosa* (6749)
(4) *Bacillus pumilus* (N.C.I.B-8982)
(5) *Candida albican*
(6) *E. coli* (N.C.I.B-8134)

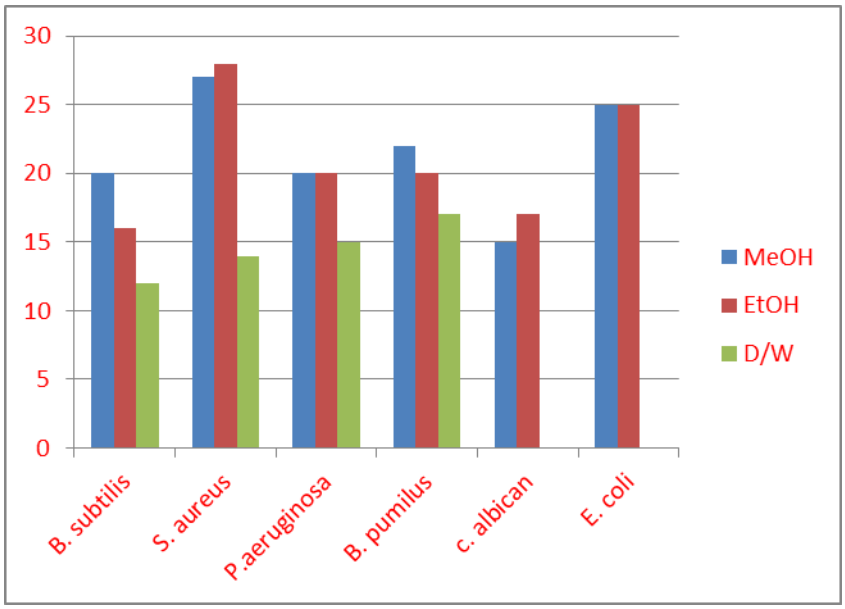

Figure (5) antibacterial activity of Neem (*Azadirachta indica* A. Juss.) leaves extracts from methanol, ethanol and distilled water (D/W)

In this study, we have shown that methanol, ethanol and D/W extracts of *A. indica* (Neem) leaves to exhibiting antibacterial activity against all tested bacterial strains. Several studies had been performed to investigate the antimicrobial activity of neem leaf extract and their results were almost similar to our results. One of these studies is the report of Okemo *et al* (2001) who stated that crude extract of neem plant was very effective against *S. aureus* and *E. coli.* Furthermore, Maragathavalli and his co-authors (2011) studied the antimicrobial activities of ethanolic extracts of neem leaves in various concentrations against pathogenic bacteria.

Conclusion

Microorganisms are becoming resistant more quickly than new drugs are being found. Thus, future research in antimicrobial therapy may focus on finding how to overcome resistance to antimicrobials, or how to treat infections with alternative means. Many of plants have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms. So, it is worthwhile to study plants and plant products for activity against resistant bacteria. Some of the best news is that neem may help in the search for prevention or a cure for AIDS may possibly be treated by ingesting neem leaf extracts or the whole leaf or by drinking a neem tea. Low doses of neem leaf extracts have sedative effects. It also reduces anxiety and stress (Bhowmik Debjit *et al*., 2010).

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