PHYTOCHEMICAL SCREENING OF GEMMOTHERAPEUTICALLY TREATED NEEM AND NATIVE NEEM: AN EXPERIMENTAL STUDY TO DETERMINE THEIR POTENTIAL MEDICINAL ROLE

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ABSTRACT

Background and Objectives: Neem belongs to the plant family "Maliace". It is used in herbal medicine for the treatment of various diseases. The objective of this study was to perform phytochemical screening and determine the biological activities of Gemmotherapeutically Treated Neem Extract (GTNE) and Native Neem Extract (NNE) and evaluate their potential medicinal use.

Methodology: Gemmotherapeutically treated neem extract (GTNE) and native neem extracts (NNE) were investigated for their chemical constituents and biological activities.

Results: Different phyto-constituents like glycosides, flavonoids, tannins, steroids, alkaloids and saponins were found to be present in GTNE and NNE, while anthraquinones were not detected. Both extracts showed strong inhibitory activities against S. aureus, Bacillus subtillus, E. coli, Aspergillus niger and Candida albicans. Both extracts also showed some inhibitory effect against earth – worm.

Conclusions: Biologically active ingredients of both GTNE and NNE show antibacterial and antifungal effects, suggesting the potential medicinal role of these substances which should be studied and explored further.

Key Words: Phytochemical screening, GTNE, NNE, Medicinal Role.

INTRODUCTION

Plants not only beautify the expanding world but also provide an irretrievable sort of integrity of animals including human.Different parts of medicinal plants and their extracts were used to treat different diseases. Methanolic Neem Leaves Extract (MNLE) when combined with cisplatin in chemotherapy may relieve cisplatin – induced damage and oxidative stress in liver.[1] About 80 percent of the world population relies on the use of traditional medicine, which is predominantly based on plant material.[2] It is heartening to say that traditional plant medicine has now led to several therapeutically and industrially useful preparations and compounds. This has encouraged scientists in exploring more information about the medicinal plants. There is a growing global awareness of using non-toxic plant products as traditional medicine. These are also used as a natural blue print for the development of new drugs.[3] Plants do not have immune systems directly comparable to that of animals. Plants produce a wide range of selective antibacterial and anti-fungal compounds to defend themselves.[4,5]

Neem is an evergreen tree cultivated in various parts of the sub-continent. Every part of tree has been used as traditional medicine. Neem belongs to family Maliace, genus Azadirachta, indica. More than 135 compounds have been isolated from different parts of neem. These compounds have been divided into two major groups isoprenoids and non-isoprenoids. The isoprenoids include di-terpenoids and tri-terpenoids, while the non-isoprenoids include proteins, carbohydrates, flavonoids, glycosides, tannins etc. Many modern drugs are also developed from neem. With the passage of time, the good use of centuries old knowledge on neem, has achieved modern approaches towards drug development.For last few years, there has been an increasing trend and awareness in neem research. Presence of flavonoids, alkaloids, glycosides etc provide the medicinal properties of neem.[6] Dry Neem Leaves (DNL) at an amount of 50 gm /16 sq ft have been considered as standard to reduce the ammonia level of litter along with its bad smell. It may be economical where biochemical products are not available or ignored by the farmers.[7]

Neem extracts have been used for its biological activity as well as in liver diseases like hepatitis.[8,9] A. indica has shown antimicrobial activity over a wide range of organisms.[10] A. indica originated in Sri-Lanka, India and Burma.[11] It is now grown in Bangladesh, Cambodia, Nepal and in many other countries around the world.[12] In Sri Lanka, the seed paste is used in anti-lice shampoos.[13] Seed oil is used against worm infect-

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ion in humans and livestock, and also for chronic forms of skin diseases. Neem oil is used to manufacture soap, the use of this safeguards the skin from microbial infection. It has a natural insecticide effect.[14]

The phyto-chemical screening is to isolate various constituents of the plants for assessing their biological activity. The medicinal value of plants is due to the presence of particular chemical substances that have a definite physiological action on living system. The most important are alkaloids, glycosides, saponins, flavonoids, steroids, anthraquinone and tannic acid. Gemmotherapy is a new form of herbal medicine using extracts of embryonic tissues from fresh plant, such as young buds, shoots, leaves and rootlets. They are collected in spring season because in spring season they are in peak life cycle stage. They contain high concentration of nutrients, vitamins and enzymes, released just at this time of year.[15]

Neem is versataile medicinal plant and the unique source of various types of compound having diverse chemical structure. Very little work has been done on biological activity and medicinal applications of gemmotherapeutically treated neem.[6] Most of the studies have isolated the majority of compounds found in azadirachta indica.[16,17] Most of the compounds which were isolated from neem, (azadirachtin) are vey toxic.[16] Other less toxic compounds include primary metabolites and secondary metabolites.[18,19] The phytochemical screening and biological activity testing of GTNE has not been carried out before. This new kind of treatment (gemmotherapy) was applied on neem in the current study. The main aim of this study was to perform phytochemical screening and determine the biological activities of GTNE and NNE to evaluate their potential medicinal use.

Table 1: *Composition of GTNE and NNE.*

Phytoconstituents	*% age of NNE*	*Mean ± SD*	*% age of GTNE*	*Mean ± SD*
Crude Alkaloid	4.00	4.133 ± 0.231	5.00	5.067 ± 0.115
	4.00		5.00	
	4.40		5.20	
Crude Saponin	5.00	4.967 ± 0.058	6.00	6.133 ± 0.115
	4.90		6.20	
	5.00		6.20	
Crude Steriod	3.00	3.033 ± 0.058	4.90	4.833 ± 0.058
	3.10		4.80	
	3.00		4.80	
Crude Flavonoid	2.50	2.550 ± 0.050	3.90	3.900 ± 0.100
	2.55		3.80	
	2.60		4.00	
Crude glycoside	4.50	4.500 ± 0.100	5.60	5.500 ± 361
	4.40		5.80	
	4.60		5.10	
Crude cardicglycoise	5.00	5.000 ± 0.000	6.00	6.00 ± 0.000
	5.00		6.00	
	5.00		6.00	
Crude Tannins	0.60	0.643 ± 0.040	0.80	0.827 ± 0.023
	0.65		0.84	
	0.68		0.84	

Table 2: *Effect of chloramphenicol against Staphylococcus aureaus, Bacillus subtillus, E. coli.*

Drug 20 µg	*Zone of inhibition*		
	S. aureus	*B. subtillus*	*E. coli*
Chloramphenicol	18 mm	14 mm	12 mm

MATERIALS AND METHODS

Plant Material and Extraction

Fresh growing shoots and leaves and mature leaves of neem were collected from Ayub Agriculture Research Institute, Jhang Road, Faisalabad. The plant materials were identified by the taxonomist, Department of Botany, University of Agriculture, Faisalabad Pakistan.

Native Neem Extract

The plant extract was prepared by the following procedure as per pre-established guidelines with some modifications.[20] Mature leaves of neem were collected and washed with distilled water. The leaves were completely dried at a shady place and grinded in a herbal grinder. A measured amount of powdered material was soaked in alcohol (70% ethanol). It was filtered by using filter paper after a month. The filtrate was concentrated in a rotary evaporator. The remaining alcohol was evaporated in incubator at 60°C to achieve maximum evaporation of alcohol. The amount of ex-

tract was measured in terms of per gram of crude powder.

Gemmotherapeutically Treated Extract

The young growing shoots and leaves to be used were cleaned and weighed. The dry weight of plant material was determined by drying it at 105°C in an oven till constant weight was achieved. The measured amount of plant material was blended in an equal mixture of alcohol and glycerin. The quantities were calculated so that the weight of this mixture would be 20 times that of an equivalent amount of dried sample. This mixture was left to stand for one month at room temperature under specific precautions by time to time shaking to promote maceration process, followed by filtration and made concentrated under constant pressure. After standing for a further 48 hours, it was filtered one more time. The filtrate was concentrated in rotary evaporator till the alcohol was evaporated. The amount of extract in glycerine was calculated on per gram of dry weight crude powder as described in a study.[15]

Phyto-chemical Tests

The GTNE and NNE was used to test the presence or absence of alkaloids, glycosides, sapoinins, flavonoids, steroids, anthraquinones and tannic acid according to pre-established procedures[21] including the measurement of secondary metabolites in crude form.

Table 3: *Effect of GTNE, NNE and different phytoconstituents against Staphyloccus aureaus.*

Extracts / Phytoconstituents	*50 mg Dose*	*Mean ± SE*	*75 mg Dose*	*Mean ± SE*
	Size of Zone of Inhibition (mm)		*Size of Zone of Inhibition (mm)*	
Crude GTNE	22.00	22.00 ± 0.000	24.00	23.933 ± 0.115
	22.00		23.80	
	22.00		24.00	
Crude NNE	19.10	19.133 ± 0.058	20.00	20.467 ± 0.416
	19.10		20.80	
	19.20		20.60	
Crude Glycoside	18.00	18.100 ± 0.100	19.00	18.967 ± 0.058
	18.10		19.00	
	18.20		18.90	
Crude Flavonoid	19.00	19.433 ± 0.451	20.60	20.800 ± 0.200
	19.40		20.80	
	19.90		21.00	
Crude Tannins	16.00	16.00 ± 0.00	17.50	17.500 ± 0.000
	16.00		17.50	
	16.00		17.50	
Crude Steroid	15.10	15.100 ± 0.000	16.00	16.00 ± 0.000
	15.10		16.00	
	15.10		16.00	
Crude Alkaloid	14.10	14.167 ± 0.115	15.00	14.700 ± 0.608
	14.30		15.10	
	14.10		14.00	
Crude Saponin	13	13.00 ± 0.265	14.1	14.033 ± 0.058
	13.5		14	
	14		14	

Microorganisms

The pathogenic strains of *E. coli*, *Bacillus subtilus* and *Staphylococcus aureus* for antibacterial test; and, *Aspergillus niger* and *Candida albicans* for antifungal tests were used. These strains were obtained from bacterial and fungal stocks, of Department of Microbiology, University of Agriculture, Faisalabad for bacterial sensitivity test.[22] Agar was prepared, and for anti fungal activity potato dextrose agar was prepared.

Antimicrobial Sensitivity Test

Two different concentrations (50 mg and 75 mg) of GTNE and NNE and different phyto-constituents were prepared by disc diffusion method on agar plates in triplicates following the standard procedures.[23,24]

For the anti-microbial activity test, the glass Petriplates of medium size (9 cm) were used. The Petri plates used were already sterilized and 15 ml of medium was allowed to sets, forms gel on cooling, making a layer of 2 – 3 mm thickness in each plate.

The Muller Hinton[22] agar plates and potato dextrose agar plates were inoculated with inoculums of 10^6 size, a sterile swab is dipped in inoculums. The

agar surface plates are streaked in three directions. The wholmen filter paper No. 1 with 5 mg and 75 mg of extracts and phytoconstituents were dried and placed at agar surface with the help of sterile forceps. The Muller Hinton agar plates were incubated at 32°C for 48 hours for anti bacterial test and the anti fungal test the potato dextrose agar plates were incubated 28°C for 10 – 14 days. The anti-bacterial and anti-fungal activities were measured as indicated by clear zones of inhibition. Ampicillin was used as positive control for bacteria and grisofulvin was used for fungal test.

Table 4: *Effect of GTNE, NNE and different phytoconstituents against Bacillus subtillus.*

Extracts / Phytoconstituents	*50 mg Dose* *Size of Zone of Inhibition (mm)*	*Mean ± SE*	*75 mg Dose* *Size of Zone of Inhibition (mm)*	*Mean ± SE*
Crude GTNE	20.00	20.267 ± 0.643	22.00	22.600 ± 0.529
	21.00		23.00	
	19.80		22.80	
Crude NNE	18.00	18.133 ± 0.321	19.00	19.667 ± 0.577
	18.50		20.00	
	17.90		20.00	
Crude Glycoside	17.00	17.00 ± 0.000	18.00	18.500 ± 0.500
	17.00		18.50	
	17.00		19.00	
Crude Flevonoid	19.00	19.00 ± 0.00	19.50	19.367 ± 0.321
	19.00		19.60	
	19.00		19.00	
Crude Tannins	16.00	16.167 ± 0.289	17.50	17.567 ± 0.058
	16.50		17.60	
	16.00		17.60	
Crude Steriod	14.10	14.100 ± 0.00	15.50	15.200 ± 0.265
	14.10		15.00	
	14.10		15.10	
Crude Alkaloids	15.50	15.500 ± 0.00	16.10	16.100 ± 0.100
	15.50		16.00	
	15.50		16.20	
Crude Saponin	15.50	13.833 ± 1.443	14.10	14.100 ± 0.100
	13.00		14.00	
	13.00		14.20	

RESULTS

The phytochemical screening revealed the presence of alkaloids, saponins glycosides, flavonoids, steroid, anthraquinone and tannic acids in both extracts investigated. It was observed their alkaloids, saponins, glycosides flavonoids, steroids and tannic acids were present; while anthraquinone was absent in both extracts. The percentage of all these phyto-constituents were also measured as shown in Table 1. The size of zone of inhibition with GTNE and NNE against *S. aureus* is shown in table 2.

Antimicrobial activity in terms of antibacterial and antifungal activity was also studied. Tests for antibacterial activity revealed that GTNE, NNE and different phytoconstituents (crude glycosides, crude flavonoides, crude tannins, crude steroids, crude alkaloids and crude saponins) that inhibited the growth of *S. aureus*. Among both extracts, GTNE 50 mg and 75 mg concentrations showed good activity as compared to the correspondingly same doses of NNE (50 mg and 75 mg) as shown in Table 3. The results revealed that the higher concentration of extracts showed higher activity as compared to lower concentration of extracts.

Among the phytoconstituents, the crude saponins showed least anti *S. aureus* activity while crude flavonoids revealed maximum anti *S. aureus* activity. The size of zones of inhibition are shown in Table 3.

The anti *B. subtillus* activity was also detected with GTNE, NNE and different phytoconstituents. The results showed that among the extracts, GTNE was more effective as compared to NNE as shown in Table 4.

Anti *B. subtillus* activity were also detected with different phyto-constituents. Table 4 showed that among phyto-constituents crude saponins were least effective while crude flavonoids were most effective against *B. sbutillus*. The higher concentrations of phytoconstituents gave good zones as compared with less concentration of phytconstituents.

Tests for Anti *E. coli* activity revealed that GTNE,

NNE and different phytoconstituents inhibited the growth of *E. coli*. The anti *E. coli* activity was detected at 50 mg and 75 mg concentrations of extracts and phytoconstituents. Both the concentrations of GTNE, NNE and different phytoconstituents inhibited the growth of *E. coli* as shown in Table 5.

The results showed that GTNE is more effective as compared to NNE. Among phytoconstituents crude flavonoids were more effective while crude saponins were least effective as shown in Table 5.

The results revealed that all these three strains of bacteria were sensitive against +ve control chloramphenicol. The µg concentrations of chloramphenicol inhibited the growth of *S. aureus*, *B. subtillus* and *E. coli* as shown in Table 2. The results showed that chloramphenicol gave good results against *S. aureus* as compared to *B. subtillus* and *E. coli*.

Table 7; shows that GTNE, NNE and different phytoconstituents showed inhibitory effect against *A. niger*. The zones were measured against both extracts. Among the extracts GTNE was more effective and gave bigger zones of inhibition as compared to NNE. According to Table 7 crude saponins is least effective while crude flavonoid is most effective among phyto-constituents. The 2 concentrations (5 mg and 75 mg) of extracts and phyto-constituents were tested against *A. niger*. The higher concentrations of extracts and phyto-constituents showed good results as compared to lower concentrations as shown in Table 7.

Table: 5: *Effect of GTNE, NNE and different phytoconstituents against E. coli.*

Extracts / Phytoconstituents	*50 mg Dose*	*Mean ± SE*	*75 mg Dose*	*Mean ± SE*
	Size of Zone of Inhibition (mm)		*Size of Zone of Inhibition (mm)*	
Crude GTNE	24.00	24.000 ± 0.000	26.00	26.000 ± 0.000
	24.00		26.00	
	24.00		26.00	
Crude NNE	22.00	20.700 ± 2.339	23.00	23.067 ± 0.058
	22.10		23.10	
	18.00		23.10	
Crude Glycoside	17.80	18.567 ± 1.242	18.90	18.967 ± 0.058
	17.90		19.00	
	20.00		19.00	
Crude Flavonoid	20.00	19.000 ± 1.732	22.00	22.00 ± 0.000
	20.00		22.00	
	17.00		22.00	
Crude Tannins	17.00	16.667 ± 0.577	18.40	18.400 ± 0.00
	17.00		18.40	
	16.00		18.40	
Crude Steroid	15.90	15.300 ± 1.127	17.50	17.500 ± 0.000
	16.00		17.50	
	14.00		17.50	
Crude Alkaloids	14.00	14.000 ± 0.000	15.40	15.400 ± 0.000
	14.00		15.40	
	14.00		15.40	
Crude Saponin	14.00	14.033 ± 0.058	15.00	15.000 ± 0.00
	14.00		15.00	
	14.10		15.00	

GTNE, NNE and different phyto-constituents showed inhibitory effect against *C. albicans* as shown in Table 8. The zones of inhibition were measured against two different concentrations (50 mg, 75 mg) of extracts and different phyto-constituents. The results revealed that higher concentration of extracts and phyto-constituents showed more effective results as compared to low concentration. Among the extracts, GTNE gave bigger zones of inhibition as compared to NNE.

Table 6, showed that µg concentrations of grisofulvin showed very effective results against *A. niger* and *C. albicans*. Both these fungal strains were sensitive against grisofulvin.

Table 6: Effect of Grisofulvin against *Aspergillus niger*, *Candida albicans*.

Drug 20 µg	*Zone of Inhibition*	
Grisofulvin	*A. niger*	*C. albicans*
	17 mm	20 mm

DISCUSSION

The results from present study have highlighted the scientific basis for traditional use of GTNE in the treatment of some ailments. GTNE, NNE and different phyto-constituents are reported to have antibacterial and anti fungal activity. GTNE showed better results as compared to NNE. Among the phyto-constituents, flavonoids are reported to have strong inhibitory effect against E. coli, Bacillus subtilus, Staphlococcus aureus, Aspergius niger and Candida albicans. The zones of inhibition observed in antibacterial and antifungal tests were also compared with positive control. Another study also reported that flavonoids inhibit the growth of bacteria and fungi.[25]

The present results are also in line with another study according to which 10 percent chloroform extract of neem leaves were shown to exhibit inhibitory effect against Streptococcus, E. coli and S. aureus.[26] Zone of inhibition of neem seed extract has been shown against bacillus subtillus.[8] It has been reported that some inhibitory concentration of azadirachta indica completely wiped out the staphylococcus aureus and candida albicans and for staphylococcus aureus, the killing was both dosage and time dependent.[27]

It is ***concluded*** that both GTNE and NNE have antibacterial and antifungal effects to explore their medicinal role.

Investigation must also be carried out to determine the possible toxicities of GTNE as determined in case of NNE, that allow use of neem in fair quanty without app-aren't hazardous consequences.[28] An extensive research and developmental work should be under-taken on GTNE for its better economic and therapeutic utilization.

Table 7: *Effect of GTNE, NNE and different phytoconsituents with 50 & 75 mg dose against Aspergillusniger.*

Extracts / Phytoconstituents	*50 mg Dose*	*Mean ± SE*	*75 mg Dose*	*Mean ± SE*
	Size of Zone of Inhibition (mm)		*Size of Zone of Inhibition (mm)*	
Crude GTNE	19.00	19.000 ± 0.000	20.40	20.400 ± 0.00
	19.00		20.40	
	19.00		20.40	
Crude NNE	17.50	17.500 ± 0.000	18.40	18.400 ± 0.00
	17.50		18.40	
	17.50		18.40	
Crude Glycoside	14.00	14.00 ± 0.00	15.00	15.00 ± 0.00
	14.00		15.00	
	14.00		15.00	
Crude Flevonoid	16.00	15.933 ± 0.058	17.00	16.967 ± 0.058
	15.90		16.90	
	15.90		17.00	
Crude Tannins	13.00	13.000 ± 0.00	14.10	14.133 ± 0.058
	13.00		14.10	
	13.00		14.20	
Crude Steriod	12.00	12.00 ± 0.00	13.10	13.067 ± 0.058
	12.00		13.10	
	12.00		13.00	
Crude Alkaloids	15.00	15.00 ± 0.000	16.10	15.867 ± 0.404
	15.00		16.10	
	15.00		15.40	
Crude Saponin	13.90	13.933 ± 0.058	15.10	15.300 ± 0.173
	13.90		15.40	
	14.00		15.40	

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Table 8: *Effect of GTNE, NNE and different phytoconsituents against Candida albicans.*

Extracts / Phytoconstituents	*50 mg Dose*	*Mean ± SE*	*75 mg Dose*	*Mean ± SE*
	Size of Zone of Inhibition (mm)		*Size of Zone of Inhibition (mm)*	
Crude GTNE	20.00	20.000 ± 0.000	22.00	22.000 ± 0.00
	20.00		22.00	
	20.00		22.00	
Crude NNE	18.00	18.000 ± 0.00	19.50	19.500 ± 0.00
	18.00		19.50	
	18.00		19.50	
Crude Glycoside	15.00	15.000 ± 0.000	15.90	15.900 ± 0.00
	15.00		15.90	
	15.00		15.90	
Crude Flavonoid	16.10	16.233 ± 0.153	17.50	17.500 ± 0.00
	16.20		17.50	
	16.40		17.50	
Crude Tannins	14.00	14.000 ± 0.000	15.00	15.033 ± 0.05
	14.00		15.10	
	14.00		15.00	
Crude Steriod	13.00	13.033 ± 0.058	14.10	14.267 ± 0.153
	13.10		14.40	
	13.00		14.30	
Crude Alkaloid	15.90	15.900 ± 0.00	17.00	17.00 ± 0.000
	15.90		17.00	
	15.90		17.00	
Crude Saponin	13.50	13.500 ± 0.00	14.80	14.567 ± 0.208
	13.50		14.50	
	13.50		14.40	

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