Australian Journal of Basic and Applied Sciences, 1(4): 801-812, 2007
ISSN 1991-8178

Chemical Composition and *'In vitro'* Antiviral Activity of *Azadirachta indica* A. Juss (Neem) Leaves and Fruits Against Newcastle Disease Virus and Infectious Bursal Disease Virus

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Abstract: Leaves, seeds and seed kernels of *Azadirachta indica* A juss (Neem) were studied for their chemical properties; moisture, ash, crude protein, crude lipids and total carbohydrates. GC/MS of the volatile components produced by simultaneous hydrodistillation and solvent extraction of each plant organs under investigation were carried out. GLC of the sterol and hydrocarbons fraction as well as the fatty acid fraction were studied for the plant organs. Antiviral activity of successive extractives of each of the plant organs were evaluated against Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) in culture of VERO cell and in chicken embryos.

Keywords: *Azadirachta indica*, Neem, Newcastle disease virus, Infectious bursal disease virus, Antiviral, Volatiles, Fatty acids, Sterols, GC/MS

INTRODUCTION

Neem (*Azadirachta indica* A juss), a Meliaceae family tree, is a hardy evergreen tree commonly found in South Asia and part of Africa. Its scientific name has been derived from the Persian word azadiracht-e-hind which means a freely growing tree of India (Dey and Mair, 1973). Neem leaves are eaten as vegetable, and twigs are used as toothbrushes. Neem is a nature's pharmacy (Vietmeyer, 1992). Today, researchers are saying that neem could be called "a wonder tree" and eventually expect it to benefit everyone on the planet. The medicinal properties of neem have been known since time immemorial. The earliest ayurvedic literature refers to the benefits of all parts of this majestic tree - fruit, leaf, bark, flower and root (Schmutterer, 2002; Subapriya and Nagini, 2005) Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex (Vietmeyer, 1992; Siddiqui *et al*., 1992; Garg *et al*., 1998; Ramesh and Balasubramanian, 1999; Koul, *et al*., 2003; Kaur *et al*., 2004; Koul, 2004; Senthil *et al*., 2006). More than 140 compounds have been isolated from different parts of neem. It's strong garlic odour (alliaceous) and its medicinal properties have been attributed to the presence of sulphur containing compounds (Nadkarni and Nadkarni, 1954; Dey and Mair, 1973; Balandrin, *et al*., 1988; Mubarak and Kulatilleke, 1990; Koul; 2004) and number of primary amines and secondary amines were detected (Atawodi and Spiegelhalder, 1994). Neem is used in treatment of various skin diseases (Dhawan and Ratnaik, 1993) and it has antibiotic properties (Sharma, 1993). Neem has been demonstrated to exhibit anti-inflammatory, antipyretic, antiarthritic (OKpanyi and Ezeukwa, 1981; Kaur *et al.,* 2004), antihyperglycaemic (Murthy *et al*., 1978), diuretic(Binde *et al.,* 1958) immunomodulatory (Upadhyay *et al.,* 1993; Arivazhagan *et al*., 2000), antiulcer (Dorababu *et al*., 2004), antimicrobial (Patel and Trivedi, 1962; Khan and Wassilew, 1987; Zeitlin *et al*., 1997; Kusumran *et al.,* 1998; Badam *et al*., 1999; Sai Ram *et a*l., 2000; Udeinya, 1993; Parida *et al*., 2002; Siddiqui *et al,* 2004), antimutagenic and anticarcinogenic potential effect (Kusamran *et al,* 1998). Neem is one of the most promising botanical insecticides at present (Dimetry, 1993; Moustafa 1993. Lowery and Isman, 1995). Its products are known to have strong pesticidal properties (Schmutterer *et al*., 1981; Schmutterer and Ascher, 1984, 1987; Hadis *et al.,* 2003, Koul, 2004), ascaricidal (Capinera and Froeba 2007) and larvicidal (Okumu *et al,* 2007). Regarding neem oil, it have reported anti-fertility, stimulate immune response (Upadhyay *et al.,* 1992), spermicidal and abortifacient effects (Riar *et al.,* 1990; 1991) and contraceptive potential (Sinha *et al.,* 1984a, b; Garg *et al.,* 1994, 1998; Sharma *et al.,* 1996; Yin *et al.,* 2004), and many other variable biological activities (Subapriya and Nagini, 2005). Neem oil is usually bitter and non-edible.

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Newcastle disease (ND) is regarded throughout the world as one of the most important diseases of poultry, not only due to the serious disease and high flock mortality that may result from some ND virus (NDV) infections, but also through the economic impact that may ensue due to trading restrictions and embargoes placed on areas and countries where outbreaks have occurred (Sharma, 1999). The immunosuppressive viral diseases, of which IBD threaten the poultry industry by causing heavy mortality and economic loss of production (Balamurugan and Kataria, 2006). Neem is traditionally being used as curative against certain fungal and bacterial diseases. However, evaluation of its antiviral properties is limited to few viruses' Viz. small pox, Fowl pox, polio and HSV as assessed by virus inhibition assay (Rao *et al.,* 1969; Rai and Sethi, 1972; Reddy and Sethi, 1974). Neem leaves have been reported to suppress HIV, Dengue virus type-2, group B Coxsackie (Upadhyay *et al.,* 1993; Badam *et al.,* 1999; Parida *et al.,* 2002). A fraction from neem oil (NIM-76) has also been reported to suppress Polio viruses (Sai Ram *et al.,* 2000).

The present study aims to investigate certain constituents of leaves, seeds, and seed kernels of *Azadirachta indica* A. juss and to evaluate their *in vitro* inhibitory antiviral activities against Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) in culture of VERO cell and in chicken embryos.

MATERIALS AND METHODS

Plant Materials:

Leaves and fresh fruits of neem *(Azadirachta indica* A. juss) were collected from the Ministry of Agriculture, Giza, Egypt in April 2006. Fresh fruits were manually separated into their seeds and hulls (kernels).

Viruses:

Live Newcastle Disease Virus (NDV) strains, Komarov and Lasota Live Infectious bursal Virus (IBDV) strain D78 were obtained from commercial available bank of strains. Strain Komarov and D78 were adapted on VERO cells (a cell line derived from African green monkey kidney) throughout seven successive passages by which the virus showed distant cytopathic effect (degeneration and floatation of the infected cells) on the 3rd day after infection.

Preparation of Oily Extracts:

Ground samples were seperately extracted sequentially with n- hexane, chloroform and methanol in a Soxhlet apparatus for 24 hrs. The solvent was evaporated under reduced pressure at 45-50^{o}C by using a rotary evaporator. The residue dried over anhydrous sodium sulphate and weighted. The extraction of leaves, seeds and seed kernels with *n*- hexane yielded 9.1%, 34.7%, 20.0%; with chloroform yielded 6.8 %, 11.3%, 10.7 %; and on using methanol yielded 65.2 %, 52.9%, 65.7% respectively.

Analytical Methods:

The following chemical properties; moisture, ash, crude protein and crude lipids were performed according to (AOAC, 1970). Total carbohydrates were determined after complete acid hydrolysis (Dubois *et al.,* 1956).The resulted acid hydrolysates were examined by PC using n-BuOH-Me_2CO-H_2O (4:5:1) (Jayme and Knolle, 1956) and aniline phthalate (Partridge, 1949) as spraying reagents. Quantitative determination of the separated sugars was carried out according to (Wilson, 1959). Total nitrogen of the investigated samples (0.3g) was determined by adopting the usual micro-Kjeldahl's method (AOAC, 1970). The crude protein was calculated by multiplied the total nitrogen by 6.25 (Black, 1948).

Preparation and Analysis of the Volatile Components:

The leaves, seeds and their hulls were separated, grinded and each sample was separately submitted to simultaneous hydrodistillation and solvent extraction (Macleod and Cave, 1975) using Likens-Nickerson apparatus using *n*-pentane (15 ml) as the second solvent. After cautious removal of solvent, the yield of volatiles was 35.54 %, 46.75 % and 40.65% in leaves, seeds and seed kernels, respectively. The volatiles were kept in refrigerator till analysis. The volatiles were analyzed by capillary gas chromatography coupled to mass spectrometry (CGC-MS). The separated compounds were identified by their retention times and mass spectra which were compared library NST and those reported in literature (Adam, 1989, Eight peak index of Mass Spectra, 1989).

GC/MS for the Volatile Components:

Gas chromatograph directly coupled to a mass spectrophotometer, Hewlett-Packard 5840, Finnigan with FID. Capillary column of DB-5 fused silica 30 m x 0.25 mm id., 0.25µ m thickness; carrier gas: helium at 30 ml/min.; temp. Programm: 60-220°C at a rate of 3°C/min.; ion source temperature 180°C; ionization voltage 70 eV.

Preparation of Unsaponifiable Matter (USM) and Fatty Acids (FA):

The hexane extract obtained from exhaustive extraction of 100 g of each separate sample of plant leaves, seeds and its kernel was separately saponified by refluxing with 10 % alcoholic KOH (El-Said and Amer, 1965; (British Pharmacopoeia, 1993) for three hours. The alcohol was distilled off, 50 ml water was added to the residue and the mixture was transferred to a separating funnel and shaken with ether (4´ 20 ml). The combined ethereal extract was washed with water till free from alkalinity, dried over anhydrous sodium sulphate and the solvent was distilled off. The unsaponifiable matter was obtained as sterols and hydrocarbons fraction. The aqueous mother liquor was acidified with 10% hydrochloric acid and the liberated fatty acids were extracted with ether (4 ´ 20 ml). The combined ethereal extract was transferred to a separating funnel, washed several times with distilled water till acid-free, dried with anhydrous sodium sulphate and the ether was distilled off.

Preparation of Fatty Acid Methyl Esters:

The resulted fatty acids were subjected to methylation (Harborne**,** 1984) by refluxing with 50 ml of absolute methanol and 3 ml of sulphuric acid for 2 hours. The methylated fatty acids were extracted with ether. After evaporation of ether, the fatty acid methyl esters were analyzed using GLC.

GLC of Unsaponifiable Matter:

Column: 1 % OV-17 packed column, temperature 250 °C; stationary phase dimethylsilicone fluid; detector temperature, 300 °C; injector temperature, 250 °C; carrier gas, N2, flow-rate 30 ml/min.; air flow rate, 330 ml/min.; H2 flow-rate, 30 ml/min.; detector, FID (Flame Ionization Detector); chart speed, 0.5 cm/min.; oven program: initial temperature 70°C, at rate 10°C/min., final temperature 270 °C.

GLC of Fatty Acid Methyl Esters:

Five micro liters of the trimethylsilane derivatives of the sample or authentic was injected into the gas chromatography by means of a micro syringes applying the following conditions: instrument: Hewlett Packard HP 6890 Series GC system; column: capillary column HP-Innowax Polyethylene Glycol; length, 30 m ; diameter, 530 cm, 1 mm (film thickness) ; oven: temperature program: rate, 2 °C /min.; initial temperature, 120 °C; final temperature, 240 °C ; back inlet: (splitless) temperature; 275 °C ; pressure, 12.28 psi; flow, 16.1 ml/min.; detector: flame ionization detector temperature, 300 °C; carrier gas: nitrogen flow rate, 30 ml/min; hydrogen flow rate, 30 ml/min.; air flow rate, 300 ml/min.

Antiviral Activity:

In VERO cell cultures, anti-NDV, strain Komarov and IBDV, strain D78 activity and its cytotoxicity:

These assays were performed in 9 tissue culture plates of 24 well following the roles of (Cox *et al.,* 1996). Confluent monolayers of VERO cells were infected with 5000 tissue culture infected dose fifty ($TCID_{50}$)/ 0.2 ml/well of NDV or 50000 $TCID_{50}$ of IBDV and incubated for two hours (for virus adsorption) then inoculum was decanted, followed by addition of different ten-fold concentrations of neem fractions separately (from 3 -900 µg /ml/well/each concentration) virus infectivity control and Neem fractions cytotoxicity control were done, separately. Test plates were incubated at 37 °C and 5% CO_2 for 2 days. The cytotoxic concentration fifty (CC_{50}) of neem extract and its fractions was determined as the concentration of compounds that induced any deviation of the morphology than the normal control cells in 50% of VERO cell monolayers. Antiviral inhibitory concentration of fifty (IC_{50}) of samples was assayed as the concentration of compounds that fully inhibited virus-cytopathic effect (100 $TCID_{50}$) in 50% of monolayers. Also, the therapeutic index (TI) of samples was expressed as CC_{50}/IC_{50}. The CC_{50} and $TCID_{50}$ were calculated by the method of (Reed and Muench, 1938).

In chicken embryos, anti-NDV strain Lasota and IBDV, strain D78:

Groups of 9-11 day old specific pathogen free (SPF) embryonated chicken eggs (ECE) were inoculated with 500 embryo infective dose fifty (EID_{50})/0.2/egg of NDV, strain Lasota or 50 EID_{50} of IBDV, strain D78; immediately followed by injection of different concentration of samples (from 4-400 µg /0.2 ml/egg), separately. Virus infectivity control and neem samples toxicity controls were inoculated via the chorioallantoic cavity. Test eggs were incubated for 3-4 days at 37°C and 80% humidity. The CC_{50} , IC_{50} and TI values were determined as mentioned before NDV, NDV strain Lasota. Infectivity in ECE was detected by

heamagglutinating activity of the allantoic fluids of t he inoculated eggs as measured by micro technique of the haemagglutination (HA) test (Takatsy, 1956) while IBDV, strain D78 infectivity was determined by the criterion of distension of the abdominal region, mottled necrotic or hemorrhagic liver and mortality scores in embryos.

RESULTS AND DISCUSSIONS

Chemical Analysis:

The percentage composition of leaves ash (9.5%) was higher than seeds (4.1%) and their kernels (3.9%) and the higher percentage of polymeric carbohydrates was recorded for the seeds kernels (46.7%) while, the higher crude protein percentage was found in leaves (17.4 %) than the seeds kernels (11.0%) and the seeds (9.1%). The seeds (46.0%) followed by seed kernels (37.7%) were found to contain higher percentage of total lipids than the leaves (15.9%). The results of these chromatography investigations (Table 1) revealed the presences of glucouronic acid, galactose, glucose, arabinose and xylose as structural unites of leaves, seeds and seed-hulls. However, traces of rhaminose (Table 1).

Table 1: Percentage of ash, total carbohydrate, protein, and monosaccharide composition ratio of leaves, seeds and seed-kernels of *Azadirachta indica* A. juss.

Plant	Ash	Total Carbohydrates	Proteins	Lipids	Monosaccharide Composition ratio					
					UA	Gal	Glu	Arab	Xyl	Rham
Leaves	9.5	21.1	17.4	15.9	3.9	25.2	34.6	28.2	7.0	1.1
Seeds	4..1	12.4	9.1	46.0	4.7	6.9	70.9	6.4	11.1	T
Seed-kernels	3.9	46.7	11.0	37.7	2.9	12.9	66.7	7.1	10.4	T

T. Traces (<1%), UA (Uronic acid), Gal. (Galactose), Glu. (Glucose), Arab. (Arabinose), Xyl. (Xylose), Rham. (Rhaminose).

GC/MS Analysis of Neem oil:

Substances were identified by their retention times and mass spectra which were compared with library NST and confirmed with those reported in the literatures (Adams,1989; Eight Peak Index of Mass Spectra, 1989). The chemical composition of essential oil isolated from leaves by hydrodistillation was analyzed by GC/MS. The results are compiled in Tables 2, 3 and 4 for the leaves (Table 2), seeds (Table 3) and seed kernels (Table 4) of the plant. Analysis of volatiles of leaves was determined that thirty nine compounds wer identified, which represented 86.81 % of total oil. The oil contains mainly β-caryophyllene (9.81%), bornyl acetate (6.62%), n-undecane (6.31 %) germacrene B (5.41 %) and spathulenol (5.02 %). On the other hand, fifty compounds were identified in the seeds representing 94.74 % of the volatiles. Germacrene B (11.59 %) represented the major constituent of seeds oil as well as methyl palmitoleate (7.04 %), hexadecanoic acid (4.99 %) and *trans*-caryophyllene (4.20 %). The seeds kernels's volatiles contain also germacrene B as the main constituent (14.59 %). In addition to palmitic acid (6.72%), *trans*-caryophyllene (3.21 %) and n-hexadecanoic acid (3.31 %). Thus, generally the oils were found to contain variable constituents of aromatics, esters, fatty acids, *n*-alkanes, sulfur and nitrogen compounds, and terpenoids. Differences in oil composition were observed between the reported literatures related to the chemotaxonomy effect (Kurose and Yatagai, 2005).

GLC Analysis of unsaponifiable matter (USM):

Neem seeds have higher yield of lipoidal matters Table 1. The leaves, seeds and seed kernels lipoidal matters afforded 62.47%, 70.45 % and 84.32 %, respectively of unsaponifiable matter fractions and 26.49%, 22.23 and 9.65%, respectively of fatty acids fractions. So, the percentage of unsaponifiable fraction is higher than the total fatty acids in all plant investigated organs. The percentage of unsaponifiable fraction was higher in seed kernels (84.32%), while the leaves had the highest fatty acid fraction percentage (26.49%).

Analysis of the hydrocarbons and sterol contents of the USM fractions of the lipoidal matter of leaves, seeds and seed kernels was carried out by GLC. Identification of the constituents was carried out by comparison of their retention times and coinjection with the available references compounds. Quantization was based on peak area integration. The results are compiled in Tables 5. The percentage of the identified hydrocarbons was higher than those of the identified sterols in all investigated plant organs investigated. The highest hydrocarbons percentage was found to be of leaves (60.38 %) followed by seed kernels (57.97 %) and seeds (46.81 %). While, the sterol percentage of leaves (24.56 %) and seed kernels (24.45 %) was nearly the same. β-amyrin was detected in both of leaves (3.02 %) and seeds (4.30 %), but not detected in seed kernels.

Table 2: GC/MS Analysis of volatiles constituents of *Azadirachta indica* A. juss leaves.

Identified peak No.	RT	Area %	Name
1	6.31	1.15	Hexanal
2	7.02	2.04	α-Pinene
3	7.14	0.41	Ethyl butyrate
4	8.35	0.53	2-Methyl-2,3-pentanediol
5	9.16	0.85	1-Pentanol-5-cyclopropylidene
6	9.91	1.07	β-Pinene
7	10.32	0.59	Myrcene
8	10.72	0.08	2, 4--Heptadienal
9	11.04	3.34	Limonene
10	12.39	0.08	2-Pinen-4-one
11	12.82	1.81	Isopropyl benzyl alcohol
12	14.67	3.74	*trans*-Pinocarveol
13	14.74	6.31	n-Undecane
14	14.76	1.09	3-Cyclopentene-1-acetaldehyde 2, 2, 3 trimethyl
15	15.00	3.02	*cis*-Verbenol
16	15.89	0.06	2-Undecanone
17	16.34	3.26	Terpinen-4-ol
18	19.93	6.62	Bornyl acetate
19	24.81	3.70	(2R, 3R)-3 Phenyl-1,2-butandiol
20	25.82	0.13	Ethyl propyl disulfide
21	27.33	0.46	δ-Elemene
22	27.61	1.62	α-Cubebene
23	27.88	4.70	*n*-Tetradecane
24	28.29	1.18	Longifolene
25	28.35	9.81	β-Caryophyllene
26	28.83	1.66	Eudesma-4(14), 11-diene
27	28.61	1.20	*Allo*-Aromadenadrene
28	30.12	0.41	Humulene-oxide
29	30.70	0.97	Bornyl isovalerate
30	31.11	1.80	γ-Muurolene
31	31.71	5.41	Germacrene B
32	31.99	5.02	Spathulenol
33	32.52	2.92	2, 4, 8, Trimethyl-1,2,3,4,4.5,6,6 octahydronaphthalen, 2-yl-a-2-prop-2-en-1-ol
34	33.48	1.33	Muurola-4(5), 5-diene
35	33.97	1.97	β-Eudesmol
36	34.09	0.43	2(3 H) Naphthalenone, 4, 4, 5, 6, 7, 8-hexahydro-4, 4-dimethyl-6-(1-methylethenyl)
37	34.27	0.40	1-Hexadecanol
38	34.27	3.83	2-Butanone, 3,3-dimethyl-1-(methylsulfonyl)-, O-[(methylamino)carbonyl]oxime
39	35.17	1.81	Methanone, Phenyl, 1,3,4-Trimethyl-2-(methylthiio)-3-cyclopenten-1-yl-

Table 3: GC/MS Analysis of volatiles constituents of *Azadirachta indica* A.juss seeds.

Identified peak No.	RT	Area %	Name
1	5.77	0.11	.2-Methyl-pentanal
2	5.93	0.91	Ethyl butyrate
3	6.98	1.14	2-Methyl-2,3-pentanediol
4	7.21	2.48	Octane
5	8.43	0.59	Camphene
6	9.49	1.54	β-Pinene
7	10.88	2.45	1-Nonanol
8	13.78	1.94	1,2-Epoxy-4-propanoxy-hexane
9	19.94	0.20	2-Undecanone
10	24.22	2.49	1-Tetradecene
11	24.67	0.32	Dichloromethyl propyl sulfone
12	28.23	0.31	Longifolene
13	28.62	4.20	*trans*-Caryophyllene
14	29.60	0.54	β-Farnesene
15	33.82	11.59	Germacrene B
16	34.09	0.62	3-Ethoxy-5,6,7,8-tetrahydro-1-hydroxyisoquinoline-4-carbonitrile
17	34.31	1.84	Eudesm-7(11)-en-4-ol
18	34.79	0.98	Cetene
19	35.58	2.66	1,3-Dipropionyl-2,4,6-trimethylbenzene

Table 3: Continued

20	35.72	2.50	Valeroyl pentamethyl benzene
21	35.80	2.97	2-Phenylundecane
22	36.37	1.83	2-Butanone, 3,3-dimethyl-1-(methylsulfonyl)-, O-[(methylamino)carbonyl]oxime
23	36.61	2.04	5-Eicosene
24	37.69	1.01	1,1,2,2-Tetramethyl-3-[(t-butyl)methylene]-8-oxobicyclo[4.3.0]non-4(5)-ene
25	37.92	3.59	Tetradecanoic acid
26	38.12	1.81	1-Propyldecylbenzene
27	38.43	2.71	5,6-Dihydro-2,4,6-triethyl-(4H)-1,3,5-dithiazine
28	38.71	0.38	8,9-Dihydrocyclohepta[a]phenalen-7,10-dione
29	40.54	0.06	Abieta-8, 11, 13-tridiene
30	41.02	1.00	Methanone, Phenyl, 1,3,4-Trimethyl-2-(methylthiio)-3-cyclopenten-1-yl-
31	41.72	3.15	Pentadecanoic acid, 14-methyl-, methyl ester
32	42.43	4.99	Hexadecanoic acid
33	44.16	7.04	Methyl palmitoleate
34	52.51	2.37	9-Octadecenoic acid
35	52.63	1.87	4-Octylphenol
36	52.91	1.69	Octadecanoic acid, methyl ester
37	53.21	3.96	*n*-Docosane
38	53.79	0.19	6,9,10-Trimethoxy-12H-[1]benzoxepino [2,3,4-ij]isoquinoline
39	53.99	0.85	di-2-Benzothiozole disulfane
40	54.54	0.67	12-Phenylindolo[1,2-b][2,6]naphthyridine-5,11-dione
41	54.72	0.14	7-Methlene-5,8,9-trimethyl-3-mesitylisoxazolo [4,5-c]tricyclo[3.2.1.0(2,7)]octan-9-ol]
42	54.80	0.14	Methyl 4,5-dihydro-7-oxo-8-phenyl-7H-thieno[2,3-a]quinolizine-10-carboxylate
43	54.88	1.21	2-(di (4-Hydroxy-3-methoxyphenyl)methyl)pyridine
44	54.95	1.36	N-(2,5-di-Tertbutylphenyl)phthalimide
45	54.98	0.19	Tetramethylaglycone
46	55.23	1.94	*n*-Tetracosane
47	55.44	0.09	4-(*p*-Cumylphenoxy) phthalonitrile
48	55.79	2.08	4-(*p*-Methylphenylmethasulfonamino)-6,7-dihydroxy-2-oxabicyclo[3.3.1]nonan-3-one
49	55.84	1.28	Methyl 6-Methoxy-9-(2-methoxyphenyl)-1-oxo-1H-phenalene-7-carboxylate
50	55.92	2.72	2,5-Diphenyl-3-(2-naphthoyl) pyrrole

Table 4: GC/MS Analysis of Volatiles Constituents of *Azadirachta indica* A. juss Seed Kernels.

Identified peak No.	RT	Area %	Name
1	7.69	1.97	3-Hexanol
2	8.13	2.27	3-Pentanol 2-Methyl
3	8.57	0.19	Camphene
4	12.46	0.40	n-Propyl trans-I-propenyl tetrasuflide
5	13.60	2.95	*cis*-1,4-Undecadiene
6	22.14	0.72	2-Phenylethanol
7	22.67	1.61	1,2,3-Trithiacyclohexane
8	22.87	1.77	Trithiacyclononane
9	28.77	0.32	d-Elemene
10	28.92	3.21	*trans*-Caryophyllene
11	29.02	14.59	Germacrene B
12	29.53	0.23	Valencene
13	30.13	0.71	L-Tumerone
14	30.25	1.25	Bicyclogermacrene
15	31.92	2.62	1,3-Dipropionyl-2,4,6-trimethylbenzene
16	32.02	1.63	Isospathulenol
17	32.64	6.42	1-Benzyloxymethyl-1-hydroxymethyl-2,5-cyclohexadeine
18	34.30	0.33	Methyl *trans*, *cis*-farnesate
19	34.65	2.82	6,10-Dodecadienoic acid, 3,7,11-trimethyl-, methyl ester
20	34.71	0.39	(Z)-3-(Tosylmethyl)-3-penten-2-one
21	34.82	0.09	2-Butanone, 3,3-dimethyl-1-(methylsulfonyl)-, O-[(methylamino)carbonyl]oxime
22	34.89	6.72	Palmitic acid
23	34.90	3.31	*n*-Hexadecanoic acid

Table 4: Continued

24	36.11	0.58	5-O-Benzyl-2,3-O-isopropylidene-D-ribonolactone
25	36.23	0.11	Benzodicarboxylic acid dibutyl ester
26	36.72	2.84	Methyl palmitate
27	38.47	0.29	1,2,3,4,8,9- Hexahydro- Dibenz[a,j]anthracene,
28	40.50	2.18	Methyl Octadec-10- enoate
29	40.91	1.21	2-(3,4,5-Trimethylphenyl)-3,3-(2,2'-biphenyldiyl)-2-propene
30	41.22	1.81	Methyl stearate
31	41.84	1.32	1,2-Benzenedicarboxylic acid, bis(2-methoxyethyl) ester
32	44.72	2.95	Cyclopropanepentanoic acid, 2-undecyl-, methyl ester, trans-
33	44.78	0.45	Ethyl oleate
34	48.16	0.13	6,9,10-Trimethoxy-12H-[1]benzoxepino [2,3,4-ij]isoquinoline
35	49.29	2.01	*cis*-3,5-Diethyl-1,2,4-trithiolane
36	49.76	1.86	Hexadecanoic acid, 2,3-dihydroxypropyl ester
37	53.28	2.62	2-(4-Acetylphenyl)-phenanthro[9,10-d]oxazole
38	54.14	1.53	2,4,6,8,9-Pentathiaadamantane-3-methanol, à,à,1,5,7-pentamethyl
39	54.44	0.97	Hexyl oleate
40	54.64	3.59	Pentacosane

$R_{t\ (min.)}$ = retention time in minutes
m/z mass/charge

Table 5: Gas liquid chromatography analysis of unsaponifiable matter of *Azadirachta indica* A. juss Leaves, Seeds and Seed kernels.

Peak No.	*Rt (min)	Area %			Comparable with
		Leaves	Seeds	Seed kernels	
1	8.64	2.96	2.12	1.24	Pentadecane
2	9.32	1.04	0.55	1.11	Hexadecane
3	10.42	0.21	0.23	2.14	Heptadecane
4	12.03	3.26	0.62	1.56	Octadecane
5	14.74	7.89	3.85	5.82	Nonadecane
6	16.85	12.47	7.28	21.13	Eicosane
7	18.08	1.85	1.42	0.96	Heneicosane
8	19.48	0.43	2.94	0.94	Docosane
9	20.97	1.4	4.58	2.3	Tricosane
10	21.87	8.55	7.46	9.51	Tetracosane
11	23.1	5.37	8.11	7.13	Pentacosane
12	24.17	4.32	3.2	1.23	Hexacosane
13	26.89	2.49	2.55	2.72	Heptacosane
14	26.95	8.14	1.9	-	Octacosane
15	29.61	4.12	6.01	2.06	Cholesterol
16	31.23	10.2	3.24	16.35	Campasterol
17	31.91	3.54	21.55	3.73	Stigmasterol
18	34.91	6.7	2.81	2.31	β–Sitosterol
19	38.39	3.02	4.3	-	β-Amyrin

No., Number; $*R_{t\ (min)}$ retention time in minutes

Table 6: Gas Liquid Chromatography Analysis of Methyl Ester of *Azadirachta indica* A. juss Leaves, Seeds and Seed kernels.

Peak No.	*R t(min)	Area %			Comparable with
		Leaves	Seeds	Kernels	
1	15.13	0.93	-	0.36	Capric acid (C10; 0)
2	16.32	2.5	-	0.53	Lauric acid (C12; 0)
3	18.71	7.81	8.37	2.84	Myristic acid (C14; 0)
4	22.14	16.98	8.96	23.79	Palmitic acid (C16; 0)
5	23.02	9.24	0.78	0.95	Palmitoleic acid (C16; 1)
6	26.43	6.54	-	2.17	Margaric acid (C17; 0)
7	26.83	6.68	30.78	11.44	Stearic acid (C18; 0)
8	27.84	27.86	34	41.37	Oleic acid (C18; 1)
9	28.55	9.46	12.55	11.38	Linoleic acid (C18; 2)
10	29.33	3.49	0.61	1.22	Linolenic acid (C18; 3)
11	29.61	2.05	0.65	1.26	Arachidic acid (C20; 0)
12	30.74	1.22	1.66	0.57	Arachidonic acid (C20; 4)
13	33.37	0.97	0.33	0.38	Behenic acid (C22; 0)
14	33.97	1.38	-	-	Lignoceric (24; 0)

No., Number; $*R_{t\ (min)}$ retention time in minutes

Table 7: Susceptibility of NDV strain Komarov and IBDV strain D78 to tested samples and their cytotoxicity in VERO cells.

Virus	Extract	CC50 (µg/ml)	IC50 (µg/ml)	TI
NDV	ML	> 200	≤ 3	> 66
5000	HL	> 300	≤ 4	> 75
TCID50	CL	> 600	≤ 5	> 120
	MK	> 700	≤ 7	> 100
	HK	> 500	≤ 5	> 100
	CK	>700	≤ 7	> 100
	MS	> 800	≤ 8	> 100
	HS	> 300	≤ 4	> 75
	CS	> 300	≤ 4	> 75
	ML	> 200	≤ 3	> 66
IBDV	HL	> 300	≤ 4	> 75
500	CL	> 700	≤ 7	> 100
TCID50	MK	> 600	≤ 6	> 100
	HK	> 600	≤ 5	> 120
	CK	> 600	≤ 6	> 100
	MS	> 900	≤ 9	> 100
	HS	> 300	≤ 4	> 75
	CS	> 300	≤ 4	> 75

CC_{50} Toxic concentration 50; IC_{50} Inhibiting concentration 50; TI Therapeutic index; 1: Methanol extract of leaves; 2: Hexane extract of leaves; 3: Chloroform extract of leaves; 4: Methanol extract of kernels; 5: Hexane extract of kernels; 6: Chloroform extract of kernels; 7: Methanol extract of seeds; 8: Hexane extract of seeds; 9: Chloroform extract of seeds.

Table 8: Susceptibility of NDV, strain Lasota and IBDV, strain D78 to tested samples and their cytotoxicity in Embryonated chicken SPF Eggs.

Virus	Sample No.	CC50 (µg/ml)	IC50 (µg/ml)	TI
NDV	ML	300	≤ 4	> 75
500 EID50	HL	300	≤ 4	> 75
	CL	600	≤ 6	> 100
	MK	800	≤ 8	> 100
	HK	500	≤ 5	> 100
	CK	700	≤ 7	> 100
	MS	600	≤ 6	> 100
	HS	300	≤ 4	> 75
	CS	300	≤ 4	> 75
	ML	300	≤ 4	> 75
IBDV	HL	300	≤ 4	> 75
50 EID50	CL	600	≤ 6	> 100
	MK	700	≤ 7	> 100
	HK	500	≤ 5	> 100
	CK	900	≤ 9	> 100
	MS	600	≤ 6	> 100
	HS	300	≤ 4	> 75
	CS	300	≤ 4	> 75

CC_{50} Toxic concentration 50; IC_{50} Inhibiting concentration 50; TI Therapeutic index; 1: Methanol extract of leaves; 2: Hexane extract of leaves; 3: Chloroform extract of leaves; 4: Methanol extract of kernels; 5: Hexane extract of kernels; 6: Chloroform extract of kernels; 7: Methanol extract of seeds; 8: Hexane extract of seeds; 9: Chloroform extract of seeds.

Table 9: Haemagglutinating activity of NDV, strain Lasota inoculated alone or with mixture of tested samples in ECE.

ECE inoculated with different dilution of NDV							ECE inoculated with NDV dil 10-7 and Neem						
Virus dilution	HA titers in their allantoic fluids						ML conc./egg	HA-titers in their allantoic fluids					
	1	2	3	4	5	Inf./total	-	1	2	3	4	5	Inf./total
7-Oct	4096*	4096	4096	4096	-	4-Apr	400 µg	0	0	0	0	0	0/5
8-Oct	4096	4096	1024	512	-	4-Apr	40 µg	0	0	0	0	0	0/5
9-Oct	1024	512	512	256	256	5-May	4 µg	8	0	0	0	0	5-Jan
10-Oct	32	32	16	4	4	5-May	0.4 µg	8	8	8	0	0	5-Feb
11-Oct	0	0	0	0	0	0/5	40 µg	8	8	8	0	0	5-Mar

ECE: Embryonating chicken Egg *Reciprocal of allantoic fluid dilution ML: Methanol extract of leaves; Inf.: Infected; Conc.: Concentration

GLC Analysis of Fatty Acid Methyl Esters (FAME):

GLC analysis of FAME of leaves, seeds and seed kernels (Table 6) revealed the higher percentage of identified unsaturated fatty acids (51.27 %, 49.60 % and 55.49 %) than the saturated one (45.84 % and 49.09 % and 42.77 %) in leaves, seeds and seed kernels, respectively. Oleic acid was the main unsaturated fatty acid in all investigated plant organs (41.37 %, 34.00 % and 27.86 % for seed kernels, seeds and leaves), respectively. The main saturated fatty acid was palmitic of each of seed kernels (23.79 %) and leaves (16.98 %), while it was stearic (30.78%) of seeds. Variations in fatty acid composition of neem leaves, seeds and seed

kernels were observed than the reported data (Kaushik and Vir , 2000; Kaushik, 2002). The previous reported insecticidal activities of the oil (Capinera and Froeba, 2007; Senthil 2006) may be related to this bioactive constituents. Moreover, the activity may be related to the bioactive constituents and mixture of saturated, mono and di-unsaturated free fatty acids and their methyl esters as reported by Garg *et al.,* 1994 and Garg *et al.,* 1998).

In VERO cell cultures, anti-NDV, strain Komarov and IBDV, strain D78 activity of different samples of Neem alcoholic extract and its fractions:

As determined by virus-cytopathic effect inhibitory assay, demonstrated that methanol extract of leaves, hexane extract of leaves as well as the hexane and chloroform extract of seeds were completely inhibit 50,000 TCID 50 and 5000 TCID 50 of IBDV infectivity in the range 3 to 4µg/ml (Table 7), with substantial therapeutic indices of >66-75. Cytotoxicity assays indicated that the CC_{50} of methanol extract of leaves, hexane extract of leaves as well as the hexane and chloroform extract of seeds were greater than 300 µg/ml (Table 8). These results proved methanol extract of leaves, hexane extract of leaves as well as the hexane and chloroform extract of seeds possed anti-NDV and IBDV in VERO cells with absence of apparent cytotoxicity.

In chicken embryos, anti-NDV strain Lasota and IBDV, strain D78:

The activity of different samples as determined by NDV, haemagglutinating activity in allantoic fluids and IBDV, infectivity criterion in embryos, indicated that 4 µg/0.2 ml/egg of methanol extract of leaves, hexane extract of leaves as well as the hexane and chloroform extract of seeds were fully reduced the infectivity of EID_{50} of IBDV and 500 EID_{50} of IBDV (Table 8). Also methanol extract of leaves, hexane extract of leaves as well as the hexane and chloroform extract of seeds ant-NDV and IBDV-IC_{50} were ≤ 40 ng/egg and 4µg/egg, respectively. The toxicity assays of samples tested in chicken embryos indicated that at concentration of 300 µg/egg and 40 mg/egg mottled ectric liver was showed by 100 % of inoculated embryos without death on 4th day after inoculation. Thus, the recorded therapeutic indices of the four previous extracts were 75 in case of both of NDV and IBDV. Susceptibility of NDV, strain Lasota and IBDV, strain D78 to methanol extract of leaves and its cytotoxicity in embryonated chicken specific pathogen free eggs was illustrated in Table 8.

Conclusion:

Concentrations of samples ranging from 3 to 4 µg /ml had a significant inhibitory action on both NDV of 5000 $TCID_{50}$ and IBDV of 500 $TCID_{50}$ in VERO cell cultures based on their cytopathic effect assays. . *In vitro*-therapeutic indices of samples ranging from 66 to 75. In chicken embryos, Neem antiviral inhibitory concentration fifty (IC_{50}) 4µg/ egg against NDV and 1 to 4mg /egg against IBDV and its toxic concentration fifty was 300µg /egg. methanol extract of leaves, hexane extract of leaves as well as the hexane and chloroform extract of seeds were effective for inhibiting NDV and IBDV replication in VERO cells and in chicken embryos. Interestingly, *in vivo* studies should be taken to demonstrate the applicable value of samples for the prophylactic treatment of NDV and IBDV-infections. Previously the methanolic leaves extract were recorded for their antiviral effect against genital herpes infection (Zeitlin *et al.*, 1997), coxsackie B group (Badam *et al.,* 1999) and Dengue virus type-2 (Parida *et al.,* 2002) and Polio virus (SaiRam *et al*., 2000). The evidence suggested that presence of a battery of compounds besides anthraquinons, flavonoids, triterpenoids and their glycosides in methanol leaves extract and its hexane fraction as well as the hexane and chloroform of seeds.

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