Biological Activities of Characterized Isolates of n-Hexane Extract of Azadirachta Indica A.Juss (Neem) Leaves

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Abstract: The antimicrobial activities of n-Hexane extract and 5 Column Chromatography fractions, of Azadirachta indica A. juss (Neem) leaves, showed antimicrobial activities against human pathogenic bacteria (Salmonella typhi) and yeast fungus (Candida albicans). Antimicrobial properties of Azadirachta indica fractions were tested using ditchwell diffusion method. Analysis of the data projected that upon bioassay with n-Hexane neem leaves extract and 5 fractions of Column Chromatography zone of inhibition for Salmonella typhi observed was 17mm, 12mm, 5mm, 3mm and 11mm respectively. While for the yeast fungus Candida albicans the zones of inhibition, seen were 28mm, 25mm, 20mm, 3mm, 21mm and 20mm for Azadirachta indica, respectively. Results were compared to conventional drugs. GC/MS identified 45 bioactive compounds in the n-Hexane extract of Azadirachta indica leaves out of which 33 have antifungal activity. Conclusively based on the data analysis it can be said Azadirachta indica A. juss leaves extract have biological activity as good as the conventional drugs against such microorganisms.
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1. Introduction

Salmonella is a genus of rod-shaped, Gram negative non-spore forming, non predominantly motile enterobacteria with 2 to 5µm and flagella that grade in all direction (Clark M.A. et al., 1987) (Ryan K.J. et al., 2004). They cause typhoid fever, paratyphoid fever, and food borne illness.

Candida albicans is a diploid fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infection in humans (Ryan K.J. et al., 2004).

It is a parasitic fungus that can infect mouth or the skin or the intestines or the vagina and causes candidiases.Systemic fungal infection (fungemias) causes morbidity in immunocompromised patients (e.g AIDS, Cancer chemotherapy etc). Candida albicans is a commensal and a constituent of the normal gut flora of 80% of the human.

Azadirachta indica belongs to the Meliaceae family and is a drought resistant tropical to subtropical tree. For centuries, it has served mankind as "Panacea for all diseases". It is antifungal (Ramasany H, et al., 2010), antiviral (Badam L, et al 1999), contraceptive and sedative (Medicinal Properties of Neem http"//www.infinityfoundation.com/mandala/t_es/t_eorgraw_neem.htm), antioxidant (Syeda et al., 2011), antiretroviral, antimicrobial (Sewanu, et al., 2012), anti-inflammatory, active against resistant Gonorrehoea (Okeie, et al., 2009), antibacterial (Yogeswari, et al., 2012), anticancer (Raldary, et al., 2012), antitumor (Hsonuna, et al., 2011), antimalaria (Pavlović, et al.,2011), antidiabetic (Gholamreza, et al., 2012).

Analysis confirmed presence of phytochemicals like Alkaloids, Steroids, Tannins, Terpenoids, Flavonoids, Polyphenols and Saponins are found to be present in Neem Leaves. Current study is carried out to establish the biological activity of Azadirachta indica leaves.

2. Materials and Methods

Leaves of Azadirachta indica A. Juss were collected from the National Research Institute of Chemical Technology, Zaria – Kaduna State Nigeria. Authenticated sample is deposited in the Department's herbarium, of Plant Science Technology, University of Jos, Jos, Plateau State, Nigeria. The washed leaves of Neem were dried in an electric oven at 30°C for 4 days and powdered with an electric blender.

500g of Azadirachta indica A. Juss Leaves were powdered and extracted for 4 days with n-Hexane with constant shaking. Extract was dried to constant weight in a vacuum hood. Extract was applied to Column for Column Chromatography using 5 solvent systems. **1.** 100% n-Hexane **2.** 95% n-Hexane + 5%

Benzene **3.** 85% Benzene + 15% n-Hexane **4.** 20% Benzene + 20% Diethylether + 60% Methanol **5.** 100% Methanol respectively.

The organisms for present study were clinical isolates of Salmonella typhi from the National Veterinary Research Institute (NVIR), Vom, Plateau state, Nigeria. Candida albicans were clinical isolates from the Department of Pathology, ALLAMA IQBAL Medical University Lahore, Pakistan.

n-Hexane extract and its 5 Column Chromatography fractions of Azadirachta indica were bioassayed against the two organisms separately. The Potatoe Dextrose Agar plates were each swabbed on the surface with the respective organism. Using cork borer, wells were carved and 1 mg/ml of the n-Hexane neem leaves extract and 1mg/ml of each of the 5 Column Chromatography fractions were applied to the wells respectively. Antibacterial positive control was Chlorophenicol 1mg/ml and distill water was the negative control. Antifungal positive control was Fluconazol 1mg/ml and for negative control distill water was employed. The plates in triplicates were incubated at 28°C for 48hours. The diameter of zones of inhibition in millimetre of the test organisms were noted.

The GC-MS analysis of n-Hexane Azadirachta indica leaves extract and 5 Column Chromatography fractions were carried out using Agilent Technologies 7890A GC system. The detector was Agilent Technologies 5975C inert MSD with Triple-Axis Detector, with column (Polysiloxanes) 30m × 0.25m fused capillary silica tubing. Software adopted to handle mass spectra and chromatograms was National Institute of Standards and Technology MS. 2005 Library. The following temperature protocol was followed for GC/MS detection. Injection port temperature was 200°C and Helium flow rate was 1ml/min. Oven temperature was programmed from 50°C with an increase of 8°C/min to 300°C and this temperature was held for 9 minutes. The ionization voltage was 70 eV. The samples were injected in splitless mode and mass spectral scan range was set at 45-500(MHZ). The GC/MS characterized the bioactive components of the n-Hexane extract Column Chromatography fractions. The fragmentation pattern of the mass spectra were compared (head to tail) with those stored in the NIST Library. The total running time for GC was 36 minutes.

3. Results

The Results are presented as follows:

Table 1: indicates the zones of inhibition of the bacteria, while Table 2: shows the zones of inhibition for the fungus.

Table 1. Zones of Inhibition of Salmonella typhi against Azadirachta indica A. juss n-Hexane leaves extract and Column Chromatography fractions

S/N	Solvent System	Column Chromatography Fractions	Zone of inhibition (mm)
0.	n-Hexane Neem extract	Extract	17
1.	n-Hexane fraction	(CC F1)	12
2.	95% n-Hexane + 5% Benzene	(CC F2)	5
3.	85% n-Hexane + 15% Benzene	(CC F3)	3
4.	20% Benzene + 20% Diethylether + 60% Methanol	(CC F4)	11
5.	100% Methanol	(CC F5)	No Inhibition

Table 2. Zones of Inhibition of Candida albicans against Azadirachta indica A. juss leaves with n-Hexane extract and Column Chromatography fractions

S/N	Solvent System	Column Chromatography Fractions	Zone of inhibition (mm)
0.	n-Hexane Neem extract		28
1.	n-Hexane fraction	(CC F1)	25
2.	95% n-Hexane + 5% Benzene	(CC F2)	20
3.	85% n-Hexane + 15% Benzene	(CC F3)	3
4.	20% Benzene + 20% Diethylether + 60% Methanol	(CC F4)	21
5.	100% Methanol	(CC F5)	20

Table 3. shows the Gas Chromatography and Mass Spectroscopy characterized components.

Table 3. GC-MS Identified components of the Azadirachta indica A. juss Leaves extract (Compounds are listed in ascending order of Retention Time)

S/N	RT	Molecular Formula	MW	Name of Compound	Activity
1. ****	4.237	C8H10	106	p-Xylene	Antifungal, Antioxidant and antimicrobial (7)
2. ****	4.250	C9H20	128	Nonane	Antifungal and Antibacterial (12)
3. ****	4.262	C8H10	106	o-Xylene	Antifungal, Antioxidant and antimicrobial (7)
4. ****	4.806	C8H9NO2	151	Oxime-, methoxy-phenyl-	Antifungal, Antibacterial, Anticancer and Antitumor (10)
5. ****	5.845	C10H22	142	Decane	Antifungal and Antibacterial (13)
6. ****	6.389	C10H14	134	Benzene,1-methyl-2-(1-methylethyl)-	Potent antifungal Antioxidant activity (14)
7. ****	6.495	C7H8O	108	Benzyl Alcohol	Potent antifungal Active against yeasts, mold and more active against Gram-positive , than Gram-Negative Bacteria. (15)
8. ****	7.709	C9H18O	142	Nonanal	Antifungal Moderate Antibacterial activity against Gram +ve and Gram- bacteria (16)
9.****	9.179	C7H6O2	122	Benzoic Acid	Antifungal (17)
10. ****	9.391	C12H26	170	Dodecane	Enhances antifungal activity (18)
11. ****	12.213	C10H12O2	164	Phenol, 2-methoxy, 3-(3-propenyl)-	Antifungal, High level of antimicrobial activity against fish pathogens, antioxidant, and disinfectant. (3)
12. ****	12.769	C14H30	198	Tetradecane	Antifungal and Antibacterial (19)
13. ****	13.376	C10H18O3	186	Nonanoic acid, 9-oxo-, methyl ester	Potent antifungal, Antioxidant,Potent Antimicrobial (6)
14. ****	15.403	C15H24	204	.gamma.- Elemene	Antifungal, Antioxidant, and Biocidal activity (20)
15. ****	15.703	C15H24O	220	1H-Cycloprop(e)azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, {1aR-(1aalpha,4aalpha,7beta,7abeta,7balpha)}-	Antifungal, Insecticidal and larvicidal, agent (21)
16. ****	15.829	C16H34	226	Hexadecane	Antifungal, Antibacterial, antioxidant activity, (9;11)
17.	18.568	C19H40	268	Nonadecane	Cytotoxic effect,antimicrobial (11)
18. ****	18.918	C17H34O2	270	2(4H)-Benzofuranone, 5,6,7,7^{a}-tetrahydro-4,4,7a-trimethyl-	Antifungal,Antialgal effect, antioxidant, antibacterial activity (22;23)
19.	19.181	C18H36O	268	2-Pentadecanone, 6,10,14-trimethyl-	Antibacterial activity against Gram +ve and Gram-ve bacteria (16)

20. ****	19.537	C16H22O4	278	Diisobutyl phthalate	Antifungal, Antibacterial, Antiviral and Antioxidant activities (24)
21. ****	20.188	C17H34O2	270	Pentadecanoic acid, 14-methyl-,methyl ester	Antifungal, Antimicrobial (25)
22. ****	20.194	C17H34O2	270	Hexadecanoic acid, methyl ester	Antifungal,Antioxidant, hypocholesterolemic nematicide, pesticide, anti-androgenic flavour, haemolytic, 5-Alpha reductase inhibitor, potent antimicrobial activity. (26)
23. ****	20.463	C18H28O3	292	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl-4-hydroxy-, methyl) ester	Antifungal, antioxidant,(25)
24. ****	20.463	C20H40O	296	1-Hexadecen-3-ol,3,5,11,15-tetramethyl	Antifungal, Antiviral, Anti-inflammatory, Resistant gonorrhea, (8)
25. ****	20.670	C16H32O2	256	n-Hexadecanoic acid	Antifungal,Antioxidant, hypocholesterolemic, nematicide, anti-androgenic flavour, haemolytic, 5-Alpha reductase inhibitor, potent antimicrobial agent, antimalarial and antifungal. (26; 27;11)
26. ****	20.713	C16H22O4	278	Dibutyl phthalate	Antifungal,Antimicrobial agent, antimalarial and antifungal (28)
27. ****	21.064	C12H25I	296	1-iodo-2-methylundecane	Antifungal, antioxidant and antimicrobial agents. (29) Insecticidal (30)
28. ****	21.064	C20H42	268	Eicosane	Antifungal, antibacterial,antitumor and cytotoxic effects (11)
29.	22.277	C19H36O2	296	9-Octadecenoic acid (Z)-methyl ester	Antioxidant activity, Anticarcinogenic,-exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligand, dermatitigenic flavour (6;26)
30.	22.290	C19H32O2	292	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	Anticancer,Antimicrobial, Antioxidant and Hyperchloesteralemic (31)
31. ****	22.434	C20H40O	296	Phytol	Antifungal Active against Salmonella typhi, Resistant Gonorrhoea, Joint dislocation, Headache, Hernia and is a Stimulant and anti malaria (26;6;8)
32. ****	22.565	C19H38O2	298	Octadecanoic acid, methyl ester	Potent Antifungal, antimicrobial, antibacterial and at low PH. (32)
33. ****	22.959	C18H36O2	284	Octadecanoic acid	Antifungal, antitumor activity, antibacterial (11;32)

34.	23.356	C22H48	310	Docosane	Antibacterial activity (33)
35. ****	26.975	C16H22O4	278	1,2-Benzenedicarboxylic acid, heptylmethyl ester	Active. New Compound
36.	26.975	C16H22O4	278	1,2-Benzenedicarboxylic acid, ethylhexyl ester	New Compound. Activity not tested yet.
37.	26.981	C16H22O4	278	1,2-Benzenedicarboxylic acid, mono(n-octyl) ester	New compound. Inactive.
38. **** ****	27.013	C16H22O4	278	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	Antifungal, anti retroviral, anti tumor,anti diabetic anti cancer,antioxtioxidant, anti scabies anti inflammatory, potent antimicrobial agent (6;34;35)
39.	28.301	C27H56	380	Heptacosane	Antioxidant activity (36)
40.	30.059	C29H60	408	Nonacosane	Antibacterial activity (37)
41.	30.872	C30H62	422	Triacontane	Cytotoxic effect, antimicrobial (11)
42. ****	31.704	C31H64	436	Hentriacontane	Antifugal against fungal spores germination, Antioxidant, antitumor activity and antibacterial (38)
43.	33.537	C29H48O	412	Cholesta-22,24-dien-5-ol,4,4-dimethyl-	Antibacterial, trypanocidal activity, (39)
44. ****	33.618	C33H68	464	Tritriacontane	Potent antifungal, Antibacterial, antioxidant activity (40)
45.	34.291	C29H50O	414	Gamma-Sitosterol	Antioxidant, antibacterial and prophylactic activities (41)

Key: **** = Antifungal components

1. Discussions

In the current study, the bioassay of n-Hexane neem leaves extract and 5 fractions against Salmonellla typhi and Candida albicans shows activity. The antibacterial activity is moderate as inhibition zones are 17mm, 12mm, 5mm, 3mm and 11mm respectively, (Table 1:) The antifungal activity of the *Azadirachta indica* leaves is very significant, 28mm, 25mm, 20mm, 21mm and 20mm as seen in (Table 2:) GC-MS analysis identified total of 45 compounds in *Azadirachta indica* n-Hexane leaves extract viz: p-Xylene; Nonane; o-Xylene; Oxime-,methoxy-phenyl-; Decane; Benzene,1-methyl-2-(1-methylethyl)-; Benzyl Alcohol; Nonanal; Benzoic acid; Dodecane; Phenol, 2-methoxy, 3-(3-propenyl)-; Tetradecane; Nonanoic acid, 9-oxo- methyl ester; .gamma.-Elemene; 1H-Cycloprop(e)azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-,{1aR-(1aalpha,4aalpha,7beta,7abeta,7balpha)}-; Hexadecane; Nonadecane; 2(4H)-Benzofuranone, 5,6,7,7ª-tetrahydro-4,4,7a-trimethyl-; 2-Pentadecanone 6,10,14-trimethyl-; Diisobutyl phthalate; Pentadecanoic acid, 14-methyl-,methyl ester; Hexadecanoic acid, methyl ester; Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl-4-hydroxy-, methyl) ester; 1-Hexadecen-3-ol,3,5,11,15-tetramethyl; n-Hexadecanoic acid; Dibutyl phthalate; 1-iodo-2-methylundecane; Eicosane; 9-Octadecenoic acid (Z)-methyl ester; 9,12,15-Octadecatrienoic acid, methyl ester (Z,Z,Z)-; Phytol; Octadecanoic acid**,** methyl ester; Octadecanoic acid; Docosane; 1,2-Benzenedicarboxylic acid, methylheptyl ester; 1,2-Benzenedicarboxylic acid, methylheptyl ester; 1,2-Benzenedicarboxylic acid, mono(n-octyl) ester; 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester; Heptacosane; Nonacosane; Triacontane; Hentriacontane; Cholesta-22,24-dien-5-ol,4,4-dimethyl-; Tritriacontane; and Gamma-Sitosterol. Their various activities are also mentioned in Table 3: .Interestingly these components found in the n-Hexane extract differ so much from the components found in the Ethanolic extract of Azadirachta indica cited in (42), in which most of their components were Alkaloids while ours were mainly Terpenoids and Diterpenoids along with other non-polar components.

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