EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 3
298-303
Year: 2018

PHYTOCHEMICAL ANALYSIS OF NEEM LEAVES EXTRACT THROUGH GC-MS IN METHANOL SOLVENT AND ANTIFUNGAL ACTIVITIES OF NEEM LEAVES EXTRACT AGAINST DIFFERENT FUNGAL PATHOGENS

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Article Received on 02/01/2018 **Article Revised on 23/01/2018** **Article Accepted on 13/02/2018**

ABSTRACT
GC-MS chromatogram of methanolic extract of *Azadirachta indica* showed five major peaks. From the result of GC-MS it shows the presence of phytol, linolenic acid, α linolenic acid, γ linolenic acid, palmitic acid and Tridecanolic acid. 1 propanol was the major componet in the extract. Extract in methanol solvents were found to be effective to inhibition against different fungal pathogens viz, *Fusarium oxysporum*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium* and *Alternaria alternata* species. There was no zone of inhibition in *Rhizopus stolonifer* at any concentration. Zone of inhibition was seen lowest at 25 µl concentrations while at 50 µl and 75 µl concentration zone of inhibition shows progressive change in which, *Fusarium oxysporium*, *Aspergillus flavus* and *A. niger* shows zone of inhibition in the range of 14.92 mm at 50 µl and 15.62mm at 75 µl in *Aspergillus flavus*. 16.72mm at 50 µl and 21.09mm at 75 µl concentration in *Fusarium oxysporium*, 9.62mm in *Alternaria alternata* at 50 µl and 12.24mm at 75 µl concentration.

KEYWORDS: GC-MS analysis, *Azadirachta indica*, Antifungal activity, Methanol solvent.

INTRODUCTION
Azadirachta indica (A. indica) belogs to family Meliaceae, traditionally named as neem. Traditionally it is used against many therapeutic agents. Neem extract of different parts viz., leaf, bark and seeds are known to have antifungal, antibacterial, activities against different pathogenic microorganisms.[3] Antimicrobials of neem plant are efficient against different pathogens without any sideeffect. It is native to Burma and India best seen in tropical and semitropical regions. From thousands of years medicinal properties of plant have been recognized in different parts of India.[1,6] The antifungal activity of neem oil against above fungal strains showed considerably activity. Moreover, the aqueous extract of plant has been previously reported to show antifungal activity. More than 130 compounds have been identified from different parts of *Azadirachta indica* and were classified into two major groups isoprenoids like diterpenoids and triterpenoids. Condensed tannins from the bark contain gallic acid, gallocatechin, epicatechin, catechin and epigallocatechin.[18,19] Phytochemicals have gained worldwide attention for microbiologist as these are used against different pathogens and are also safe for human health.[2,4,7] The phytochemical components present in the neem are having responsible for its antifungal and antibacterial activities that includes nimbidin, nimbolinde, Azadirachtin, nimbin, gallic acid, epicatechin, catechin and margolone.[8]

MATERIALS AND METHODS
Preparation of plant extracts
Methanol Extract: 50 grams of powdered plant material was extracted with 250 ml of methanol kept on a rotary shaker for 24 h. Thereafter, it was filtered with muslin cloth and filter through sterile whatman filter paper no.1. Filtered extract was concentrated by a rotary film evaporator so as to get dry sample.

Antifungal activity test
Antifungal activity was screened by agar well diffusion method.[13] The methanol and Hexane solvent extracts of different plants were tested against fungal pathogens isolated from infected solanaceous vegetable fruits. The PDA medium was poured in to the sterile petriplates and allowed to solidify. The test fungal culture was evenly spreaded over the media by sterile cotton swabs. Then wells (6 mm) were made and in some petriplates 6 mm discs were used in the medium. Wells were prepared by using sterile cork borer. 25, 50 and 75µl of different concentration extracts were transferred in to the separate

wells. The plates were incubated at 27°C for 48-72 hrs. After the incubation the plates were observed for formation of clear incubation zone around the well and around the discs which indicated the presence of antifungal activity. The plates were done in triplicates. The zone of inhibition was calculated with standard Himedia scale. The antimicrobial activity was taken on the basis of diameter of zone of inhibition, which was measured after 7 days of incubation and the mean of three readings is presented. The presence of inhibition of the treated fungus was calculated using DMSO as standard.

GC-MS ANALYSIS OF NEEM LEAVES EXTRACT IN METHANOL SOLVENT

Collection of plant material

The leaves of *Azadirachta indica* were collected from University campus of RDVV Jabalpur

Extraction of Plant Material

Plant material (leaves, 20 gm) was extracted with 250 ml of methanol at $60C^0$ for 8hrs in Soxhlet extractor. The methanolic extracts were filtered through Whatmann No. 1 filter paper. The filtrate was evaporated to dryness at $80C^0$ and stored until further analysis.

Preparation of stock solution

The extracts were reconstituted in methanol. Methanolic extracts (1 µl) were injected for GC-MS analysis.

Gas Chromatography-Mass Spectrometry analysis

The methanolic extract of the leaves of *Azadirachta indica* was subjected to GC-MS analysis on a GC- MS Clarus 500 Perkin Elmer system comprising a AOC- 20i autosampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: Restek RtxR – 5, (30 meter X 0.25 mm) (5% diphenyl / 95% dimethyl polysiloxane), running in electron impact mode at 70 eV; helium (99. 999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 1.0 µl was employed(split ratio of 10:1); injector temperature 280 C^0. The oven temperature was programmed from 40°C (isothermal for 5 min.), with an increase of 6 C^0 / min to 280 C^0, then ending with an isothermal for 15min at 280°C. Mass spectra were taken at 70 eV; a 0.5 seconds of scan interval and fragments from 40 to 550 Da. Total GC running time was 60 minutes.

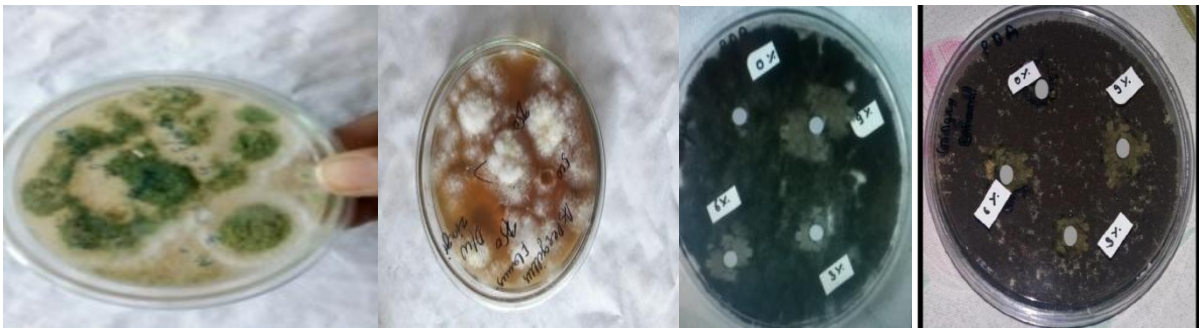
Identification of Compounds

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute of Standard and technology (NIST).The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

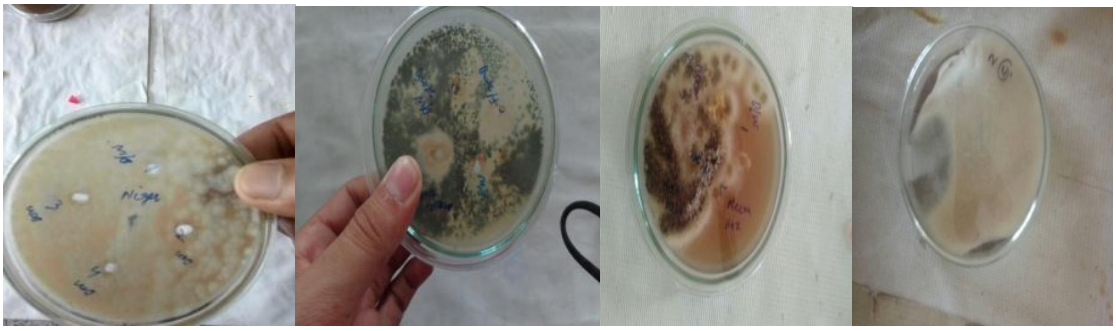
RESULTS

Table 1: Antifungal activities of neem leaves extract against fungal pathogens isolated from diseased solanaceous vegetables.

Fungal species	Mycelial growth inhibition (Zone of inhibition in mm)			
	Neem leaves extract in methanol solvent 100mg/ml			
	25µl	50µl	75µl	Solvent as negative control.
Aspergillus flavus	12.68±0.43	14.92±1.12	15.62±1.98	0
Fusarium oxysporum	12.21±1.32	16.72±0.65	21.89±1.12	0
Alternaria alternata	0	9.62±0.78	12.24±0.86	0
Penicillium nigricans.	0	8.62±1.43	13.92±1.92	0
Rhizopus stolonifer	0	0	0	0
Aspergillus niger	8.82±0.97	12.31±0.97	16.32±1.76	0
Aspergillus fumigatus	0	0	10.24±0.59	0

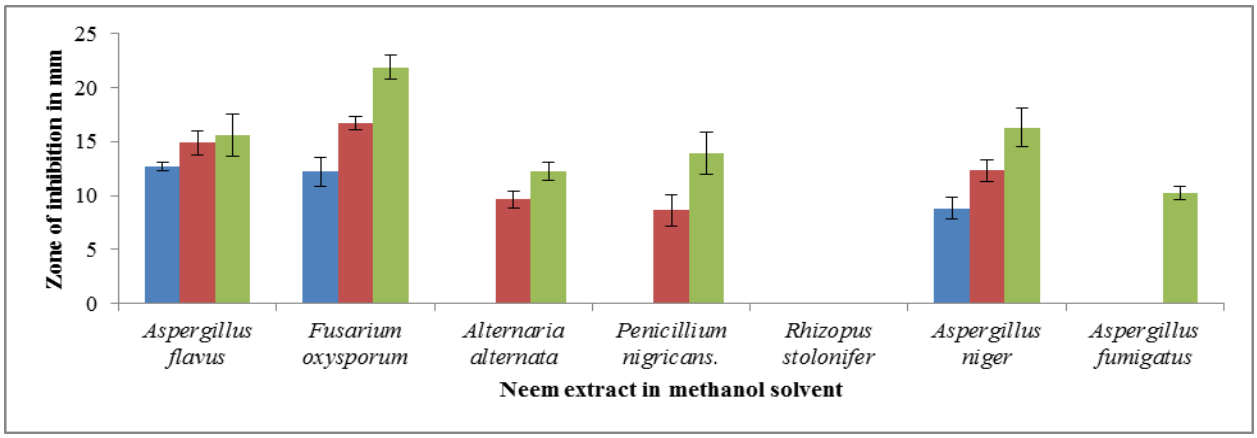


Aspergillus flavus ***Fusarium oxysporum*** ***Aspergillus niger***



Aspergillus fumigatus ***Penicillium sp.,*** ***A. allternata*** ***R.stolonifer***

Petriplates 1: Antifungal activities of neem leaves extract against fungal pathogens.



Each value is the mean of three replicates ± Standard deviation. Blue colour in graph shows zone of inhibition at 25µl, Red colour at 50µl, Parrot green colour at 75 µl concentration. Bar in the graph shows standard deviation.

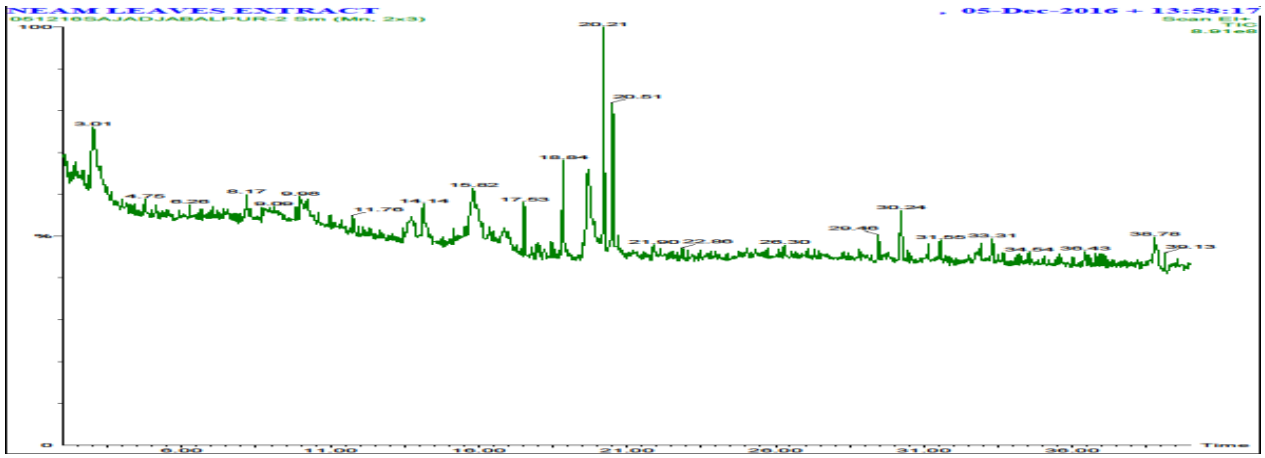


Fig. No.1: GC-MS graph of Methanolic extract of neem leaves.

Table No. 2: Compounds of neem leaves extract analysed through GC-MS analysis.

Retention time	Name of the compound	Molecular formula	Mol. Wt.	Activity
18.847	Tridecanoic acid	$C_{13}H_{26}O_2$	244.342	No activity reported
18.847	N-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.424	Nematicide 5-Alpha-Reductase-Inhibitor FLavor Hemolytic Hypercholesterolemic Pesticide Antialopecic Antiandrogenic Antifibrinolytic Antifungal
19.72	Propane-2-Methoxy	$C_4H_{10}O$	74	Antimicrobial
20.208	3, 7, 11, 15 tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.53	Cancer-Preventive Antimicrobial anti-inflammatory anti-diuretic Antioxidant, Antifungal.
20.51	9, 12, 15- Octadecatrienoic acid	$C_{18}H_{32}O_2$	278.429	Antibacterial and antifungal
3.009	1 propanol	C_3H_8O	60	Antimicrobial.

Fig. No. 2: Mass spectra of the peak having retention time 3.009.

Fig. No. 3: Mass spectra of peak having retention time 18.847.

Fig. No. 4: Mass spectra of peak having retention time 19.72.

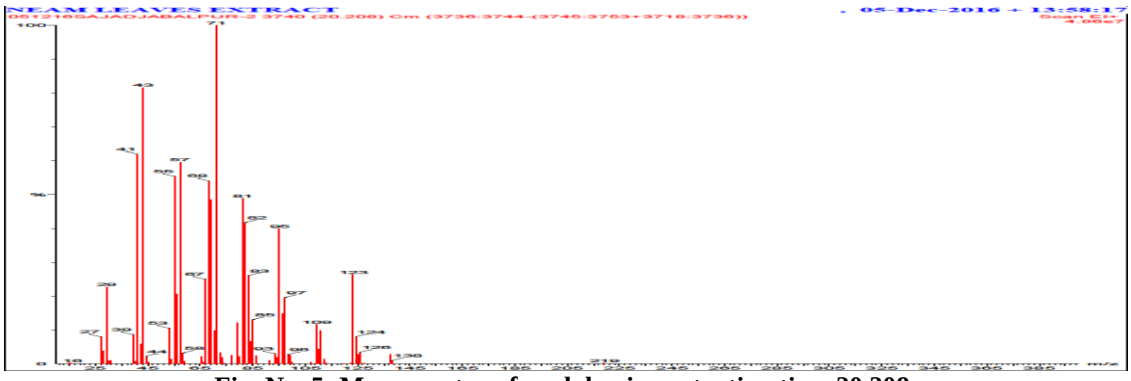
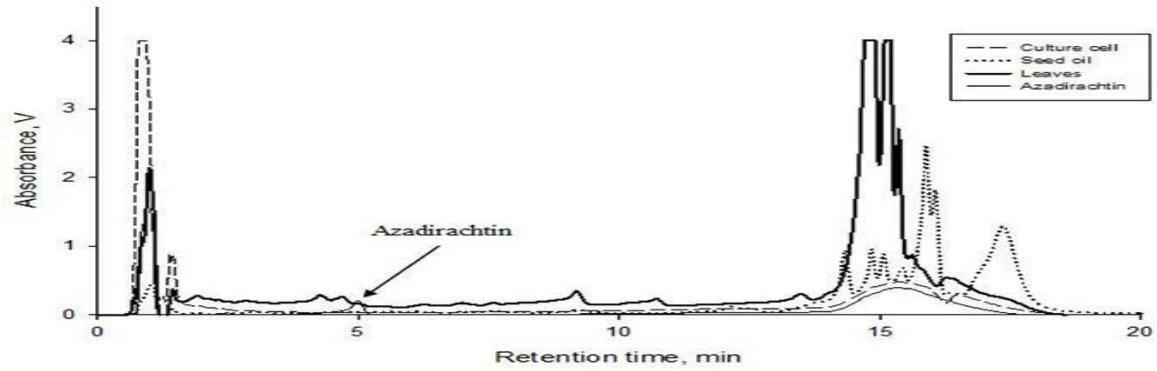


Fig. No. 5: Mass spectra of peak having retention time 20.208.



HPLC profile of leaves extract (gross solid line), seed oil extract (dotted line), cell culture extract (dashed line) and azadirachtin (thin line).

DISCUSSIONS

Neem leaves extract exhibited a capability to be fungitoxic even at high doses of inoculum, thereby indicating the possibility of its exploitation as an ideal fungi toxicant. GC-MS chromatogram of the methanolic extract of *Azadirachta indica* showed five major peaks and have been identified after by comparing the data with NIST library, results presence of five phytochemicals.It shows the occurrences of of 3, 7, 11, 15 tetramethyl-2-hexadecen-1-ol (synonym: Phytol), 9, 12, 15- Octadecatrienoic acid (synonym: Linolenic acid; α-Linolenic acid), 8, 11, 14-Eicosatrienoic acid (Synonym: Homo-γlinolenic acid), N-Hexadecanoic acid (synonym: Palmitic acid) and Tridecanoic acid (synonym: Tridecylic acid) were the major components in the extract. Phytol is reported to have antioxidant, antiallergic[17] antinociceptive and anti-inflammatory activities.[15] Recent studies have revealed that phytol is an excellent immunostimulant; it is superior to a number of commercial adjuvants in terms of long-term memory induction and activation of both innate and acquired immunity.[10] Phytol has also shown antimicrobial activity[14,16] and Staphylococcus aureus.[5] Linolenic acid is known for its potential antibacterial, antifungal[12] anti arthritic and anti-inflammatory activities[11] Lutharania *et al*., (2009) and Maruthapandian *et al*., (2011). Extract in methanol solvents were found to be effective to inhibition against different fungal pathogens viz, *Fusarium oxysporum*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium* and *Alternaria alternata* species. There was no zone of inhibition in *Rhizopus stolonifer* at any concentration. Zone of inhibition was seen lowest at 25 µl concentrations while at 50 µl and 75 µl concentration zone of inhibition shows progressive change in which, *Fusarium oxysporium*, *Aspergillus flavus* and *A. niger* shows zone of inhibition in the range of 14.92 mm at 50 µl and 15.62mm at 75 µl in *Aspergillus flavus*. 16.72mm at 50 µl and 21.09mm at 75 µl concentration in *Fusarium oxysporium*, 9.62mm in *Alternaria alternata* at 50 µl and 12.24mm at 75 µl concentration.

CONCLUSION

Neem leaves as well as other parts of this plantextract were capable of inhibiting the growth of different fungal pathogens viz *F .oxysporum*, *A.flavus*, *A.alternata*, *P.nigricans*, *Rhizopus stolonifer* and *A.fumigatus*. The extract from neem leaves had the highest antifungal activity of both, perhaps due to a higher concentration of different phytochemicals. Phytochemical constituents serve as a defense mechanism against prediction by many microorganisms, insects and herbivores.

ACKNOWLEDGEMENT

I am grateful to Prof. A.K. Pandey and Dr. Jamaludddin for giving me the opportunity and necessary infrastructure. I am thankful to the Department of Biological sciences RDVV Jabalpur for timely support by all the honorable professors. I am thankful to my lab mate also for their timely support and help especially by Akriti Shukla and Jitendra Nagpure.

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