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Analysis of aqua-organic solvents for the extraction of phytoconstituents of medicinal plants and their associated fungicidal activities

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Abstract
Background: One of the ingredients of environmental pollution is microbicides, bactericide, fungicides etc. and their overuse lead to antimicrobial resistance. This research analyses the use of phytoconstituents as fungicides, on *Alternaria solani,* a fungus using different percentage of solvents-extracts.
Methods: The extracted solvent-extracts of *Withania sominifera, Azadirachta indica and Asparagus racemosus* using different percentages of different solvents were used to test the inhibition of the pathogen at the rate of 1.73 spores/ml as spore suspension with the help of Plate reader / Spectrophotometer. For confirmation, the culture of the result was done using suitable culture media.
Result: The percentage-extraction of phytoconstituents differs with different solvents and their aqueous mixture. High percentage of extraction of phytoconstituents (mg/gm) of *Withania somnifera* leaves was obtained in 80% acetone and n-hexane and high concentration of extraction of phytoconstituents (mole/liter) of *Azadirachta indica* leaves was obtained using aqueous mixture of the acetone. 75% and 100% methanol and acetone extracts inhibited *Alternaria solani* by 83%, 96%, 92%and 95% respectively after 2 hours of incubation. 80% of acetone and n-hexane extracts of *Withania somnifera* inhibited *Alternaria solani* by 85% and 84% respectively.
Conclusion: Aqueous mixture of acetone and ethanol may be the better solvents for extraction of phytoconstituents to obtain maximum antifungal activity (here on *Alternaria solani* by *Azadirachta indica* and *Withania somnifera)* and therefore, phytoconstituents can be used as an alternate form of fungicides.

Keywords: incubation, phytoconstituents, aqueous mixture, culture medium

Introduction
Antibiotic resistance is a serious problem in many countries, both in developed and developing countries which increases mortality [1]. This is mainly due to inappropriate usage of antibiotics which leads to multi-drug resistant microbes leading to limiting the effectiveness of the current drugs [2]. Lethal effects of synthetic drugs on non-target organisms, risk of users, environmental pollution, biomagnifications in food chains etc. have been commented many times [3]. The alternate source of insecticides, pesticides, bactericides, fungicides etc. may be obtained from medicinal plants which have antimicrobial activity. Methanolic extracts from four medicinal plants, *Peganum harmala* (Zygophyllaceae), *Ajuga iva* (Labiateae), *Aristolochia baetica* (Aristolochiaceae) and *Raphanus raphanistrum* (Brassicaceae) were used to control pest, *Tribolium castaneum* (Herbst) and found effective [4]. Antimicrobial activity of ethanolic extracts of plants, *Punica granatum*, *Syzygium aromaticum*, *Zingiber officinales* and *Thymus vulgaris* against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* using agar disc at concentration of 10 mg/ml were found effective respectively while extract of Cuminum cyminum was only effective against Staphylococcus aureus [5]. The mother tincture extract of *Myroxylon balsamum* has been used for antifungal activity, for the inhibition of phytopathogenic fungi [6]. The antifungal and antibacterial activity can be observed in many fruit bearing commonly used trees such as *Tamarindus indica, Acacia nilotica, Mangifera indica* etc. [7]. The aqueous, ethanolic and ethyl acetate extracts of neem leaves, *Azadirachta indica* on human pathogens, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Candida albicans* and *Microsporum gypseum* in vitro at different concentrations, 5%, 10%, 15% and 20% yielded good result [8]. Since medicinal plants have such wonderful antifungal activities, we have undertaken a study on one fungal species, *Alternaria solani* using medicinal plants, *Withania sominifera,* and *Azadirachta indica* at different solvents to show the yield of phytoconstituents at different concentrations with minimum doze size for effective inhibition of fungi.

Materials and Methods

Leaves of *Withania sominifera*, *Azadirachta indica* and *Asparagus racemosus* were collected from Jaipur National University campus and dried. Leaves were ground and made fine powder. The powder was stored for extraction. One-gram powder each was dissolved in solvents, methanol, ethanol, acetone, n-hexane and water. The mixture was allowed to stay for two hours with often shaking and filtered using filter paper. The filtrate was evaporated, and the dry phyto-constituent was weighed. The percentage of extraction was calculated using formula; percentage of extraction = weight of extracted dry powder /dry weight of sample powder and expressed as percentage. The dried extracts were dissolved in their respective solvents and the concentration of the phyto-constituents in mg per ml was calculated. Each extract was diluted and made uniform concentration of 3.33 mg/ml and stored at -4 degree Celsius fridge for further use. The common disease-causing fungi *Alternaria solani* was isolated from *Solanum lycopersicum*, tomato plant [9] causing early blight disease, a small infected piece of leave was transferred into culture medium, potato dextrose agar. After seven days, the mycelium and spores were identified under microscopic observation. The spores were extracted from the mycelium colony and filtered using filter cloths, centrifuged the filtrate at 5000 rpm for 10 minutes. The spores were washed with water and re-centrifuged twice. Spores were counted using hemo-cytometer for making spore suspension, 1.73 $X10^6$ spores/ml [10] and stored in 50% glycerol at -4 degree Celsius. Half-gram *Azadirachta indica* powder each was dissolved in 25%, 50%, 75%, and 100% methanol and acetone respectively and the phytoconstituents were extracted.

PDB, potato dextrose broth medium was melted using sterile water and autoclaved along with Petri discs, water, test tubes etc. The inhibition study by *Withania sominifera* was conducted as follows: Seven ml of PDB, 200 microliters of spores of *Alternaria solani* and 200 microliters of solvents, methanol, ethanol, acetone and n-hexane were added in one set of test tubes and the absorbance at 595 nm was taken at 0-hours. Similarly, seven ml of PDB, 200 microliters of spores of *Alternaria solani* and 200 microliters of phytoconstituents of different solvents, methanol, ethanol, acetone and n-hexane having the concentration 3.33mg/ml were added in another set of test tubes and the absorbance at 595 nm was taken at 0-hours [11]. The absorbance was taken after everyone hour and the results were recorded for calculating the total inhibition of phytoconstituents alone (subtracted the inhibition by respective solvents). Similarly, the fungus was tested for inhibition by *Azadirachta indica* at different concentrations of methanol and acetone and the absorbance was taken at 595 nm after a gap of one hour and total of 2 hours inhibition. The spore alone in a seven ml PDB was taken as control to record the overall growth of spore in two hours.

Results

The percentage of extraction was calculated for *Withania sominifera*, *Azadirachta indica* and *Asparagus racemosus*. The percentage of extraction differs in different solvents and the maximum percentage of extraction was obtained from *Withania sominifera* when compared with the other two medicinal plants, Table 1.

Table 1: Percentage of extraction of phytoconstituents in different 80% solvents

	Methanol	Ethanol	Acetone	n-hexane	water
Percentage of extraction of *Withania sominifera*					
Percentage of extraction (%)	3	4	10	12	2
Final Concentration of extract (mg/ml)	5	6.67	16.67	20	3.33
Percentage extraction of *Azadirachta indica*					
Percentage of extraction (%)	2	4	4	3	2
Final Concentration of extract (mg/ml)	3.33	6.67	6.67	5	3.33
Percentage extraction of *Asparagus*					
Percentage of extraction (%)	2	4	5.5	5	1
Final Concentration of extract (mg/ml)	3.33	6.67	9.17	8.33	1.67

The concentration of the extracts is compared, figure 1. Very less concentration of extract was obtained in *Azadirachta indica* and very high in *Withania sominifera*.

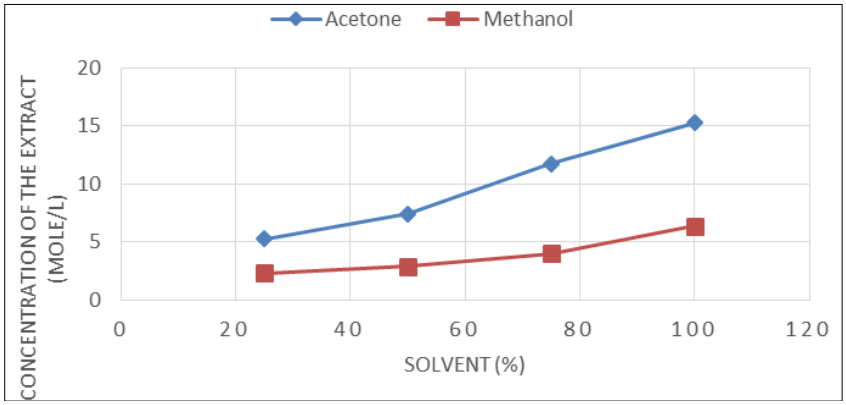

Fig 1: Comparison of concentration of extraction (mg/ml) of phytoconstituents in 80% solvents

Since *Azadirachta indica* extraction was very small amount, further analysis of extraction was done using different percentage of acetone and methanol solvents for different concentrations, table 2. Using spectrophotometer, the concentration of extract was obtained in moles/L. A graphical comparison was also done, table 2 and figure 2.

Table 2: Concentration of the *Azadirachta indica* extract in different percentage of solvents

Solvent (%)	Concentration of acetone (mole /L)	Concentration of methanol (mole /L)
25%	5.25	2.282
50%	7.438	2.881
75%	11.76	3.985
100%	15.31	6.36

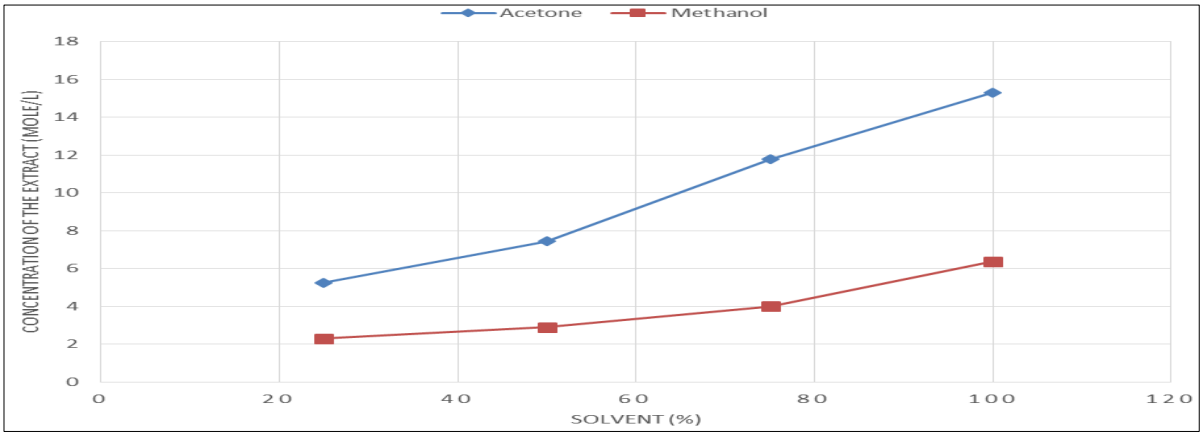

Fig 2: Comparison of *Azadirachta indica* extract indifferent percentages of solvents

Effect of extracts on *Alternaria solani.*

The extracts were used to study the inhibitory effect of phytoconstituents on *Alternaria solani*. The study was done by using *Azadirachta indica* extract of different concentrations in Methanol and acetone, table 4 and by using *Withania somnifera* in different solvent extracts, table 3.

Table 3: Inhibition of growth of *Alternaria solani* by phytoconstituents of *Withania somnifera* in different solvent extracts

	Methanol	Ethanol	Acetone	n-Hexane
One-hour inhibition by Phytoconstituent (%)	17	27	54	49
Next one-hour inhibition by Phytoconstituent (%)	19	31	31	35
Two hours inhibition by Phytoconstituent (%)	37	58	85	84

Table 4: Inhibition of growth of *Alternaria solani* by phytoconstituents of *Azadirachta indica* at different concentrations

Growth Inhibition by phytoconstituents of *Azadirachta indica* in different percentages of methanol extract				
	25%	50%	75%	100%
One-hour inhibition by Phytoconstituent (%)	8	18	40	45
Next one-hour inhibition by Phytoconstituent (%)	12	23	43	51
Two hours inhibition by Phytoconstituent (%)	20	41	83	96
Growth Inhibition by phytoconstituents of *Azadirachta indica* in different percentages of acetone extract				
One-hour inhibition by Phytoconstituent (%)	25	40	46	49
Next one-hour inhibition by Phytoconstituent (%)	28	41	46	46
Two hours inhibition by Phytoconstituent (%)	53	81	92	95

To understand the effect of inhibition visually and compare the aqua-alcoholic effect, the following figure may be helpful, figure 3.

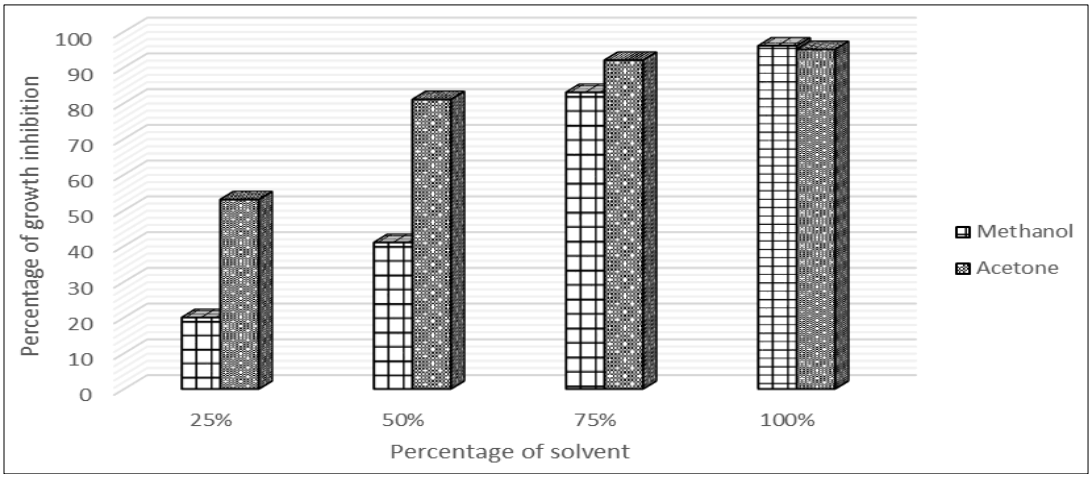

Fig 3: The inhibition pattern of different percentages of aqua-solvent extracts of *Azadirachta indica*

Discussion

The percentage-extraction of phytoconstituents depends on nature of plant parts taken for extraction, the nature and percentage of solvents chosen and the method used for extraction [12-14]. In this research, the percentage-extraction of phyto-constituents of *Withania somnifera* is better than the other two plants, table 1 which is similar to other studies as well [15] and this may be due to the presence of withaferin content [15]. The inhibition on *Alternaria solani* by *Withania sominifera* extracts of different solvents was observed and high percentage of inhibition was observed by phytoconstituents extracted using acetone and n-hexane solvents of same percentage (80%), table 3. This is similar to other research in which different species of *Alternaria* was used [16]. However, the inhibition shown by ethanol extract on *Alternaria* was higher than methanol extract of *Withania somnifera* and the inhibition by n-hexane [17] solvent extract is lower than the acetone solvent extract. This result gives some evidence of selection of ethanol and acetone as suitable solvents for extraction of phytoconstituents and for fungicidal effect of *Withania somnifera.* The result of *Azadirachta indica* was not in favor to the expected result as per the local usage of the plant by large population [18] and the existing research and literature because of the poor extraction in this research. The result of extraction of phytoconstituents using methanol is reexamined because methanol is also a better solvent for extraction.

The phytoconstituent-extract obtained from *Azadirachta indica* using all the solvents was not satisfactory, less compared to *W. somnifera* and *Asparagus*, table 1 and this was redone using different concentrations of methanol and acetone solvents, table 2 because methanol is slightly polar, and acetone is non-polar and organic solvent dissolves more in non-polar solvents [19, 20]. This view is favored by some studies and opposed by others like methanol is best solve [21], 50% acetone is best [22], in fruits the result is different [23] etc. When water alone was taken as solvent, the percentage extraction was very low, the polar phytoconstituents get extracted, table1 of this research [24] and table 1 of our research. Therefore, hydro, aqueous acetone and methanol at different percentages were taken for extraction, table 2. Improved extraction-results were obtained on all percentages of solvents of acetone [25] however, little better results from methanol solvents, table 2. The inhibition by phytoconstituents were obtained and the growth of *Alternaria* spores were inhibited by *A. indica*, inhibition with different fungal genera [26]. Surprisingly, the result is that the 25% and 50% acetone extracts have given twice inhibition percentage compared with 25% and 50% methanol, figure 2. This gives a new dimension that at a particular concentration of extraction, especially aqua-alcoholic solvents, particular phytoconstituents may dissolve in both water and alcohol which may be effective for the antimicrobial activities [27]. Similarly, very high inhibition results of both solvents at 75% and 100% concentration of solvents were obtained, similar to this study [27], table 3 and the result is highly significant however, high percentage of solvent may damage the cells membranes [28]. This research emphasis that the selection of solvents and its percentage in water is dependent on extractin and fungicidal activity and the may be recommended for mixture of solvents for better extraction.

Summary and Conclusion

The percentage of extraction of phytoconstituents differs with different solvents and its mixture. High percentage of extraction of phytoconstituents (mg/gm) of *Withania somnifera* leaves were obtained in acetone and h-hexane and high concentration of extraction of phytoconstituents (mole/liter) of *Azadirachta indica* leaves were obtained using aqueous mixture of solvents. Methanol and acetone extracts at 75% and 100% inhibit *Alternaria solani* by 83%, 96%, 92%and 95% respectively after 2 hours of incubation. 80% of acetone and n-hexane extracts of *Withania sofnifera* inhibits *Alternaria solani* by 85% and 84% respectively.

Conclusion

Aqueous mixture of acetone and ethanol may be the better solvents for extraction of phytoconstituents from medicinal plants to obtain maximum antifungal activity (here on *Alternaria solani* by *Azadirachta indica* and *Withania somnifera)* and therefore, phytoconstituents can be an alternate form of fungicides.

Acknowledgement

Authors declare that there is nothing to disclose.

Conflict of interest

Nil.

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