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BACTERICIDAL EFFECT OF PHYTOCONSTITUENTS OF MEDICINAL PLANTS

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ABSTRACT

Introduction: The antimicrobial chemicals create health hazards for all organisms and the remedy to the health hazards is switching to bio-ingredients such as phytoconstituents of plants as bactericidal agents. This research analyses the useful effect of phytoconstituents of medicinal plants as bactericides.

Methods: Leaves of *Withania sominifera, Azadirachta indica, Solanum virginianum,* and fruits and rhizome of *Solanum virginianum* and *Alocasia odora* were used to extract the phytoconstituents for the inhibition of bacteria, *Clostridium* sp*, Pseudomonas andropogonis, Bacillus sp, Pseudomonas cichorii.* The morphogenesis of growing pathogen in Nutrient broth was used to identified and measured the growth using spectrophotometer.

Results: For the extraction of phytoconstituents from medicinal plants, 80% acetone as solvent for the extraction may be suitable. All bacterial species on study can be inhibited easily by 20mg/ml concentrations of phytoconstituents of *Withania somnifera* leaves, *Azadirachta indica* leaves, S*olanum virginianum* and fruit and seeds of *Alocasia odora* rhizome. For a minimum concentration of phytoconstitutents, 5mg/ml or 10 mg/ml is effective for the inhibition. Water as solvent for extraction of phytoconstitutents for bactericidal is ineffective. *Bacillus* sp. can be inhibited easily by all concentrations and by all medicinal plants on study. *Clostridium sp.* can be inhibited by using both, Rhizome of *Alocasia odora* and seed of S*olanum virginianum*, both in 80% acetone as solvent.

Conclusion: The extracted concentration of phytoconstituents (5mg/ml, 10mg/ml and 20mg/ml) of medicinal plants in this study and the concentration of solvents (50% and 80% acetone and water) used for extraction of phytoconstituents for the inhibition of *Pseudomonas sp., Clostridium sp.* and *Bacillus* sp. vary with concentration of the phytoconstituents and concentration of the solvent used for the extraction.

KEYWORDS : Phytoconstituents, Antimicrobial, fungicide, Medicinal plants, Inhibition

1. Background

Antibiotic resistance is a serious problem in many countries, both in developed and developing countries due to increased inappropriate use, ineffectiveness and human mortality [1, 2]. Alternative to insecticides, pesticides, bactericides, fungicides etc. may be the extracts of medicinal plants which have antimicrobial activity [3, 4]. The antimicrobial activity of phytochemicals can be evaluated with the help of antibiotic susceptibility and resistant ability of microorganisms [5]. The phytoconstituents are usually considered to play a major role in defense reactions of plants against infections by pathogens [6]. For example, the phytoconstituents of Ethanolic extract of leaves of *Artemisia annua L.* showed that the extract was bactericidal at concentration 250mg/ml. The ethanolic extract of leaves of *Artemisia annua* inhibited the growth of *Staphylococcus aureus, Klebsiella pneumoniea, Candida albicans, Escherichia coli, Pseudomonas auroginosa* and *Streptococcus faecalis* [7]. Antibacterial activity of the aqueous and organic solvent extracts of the leaves of *Pavetta indica* were put to test against *Bacillus subtilis*, *Escherichia coli* and *Saccharomyces cerevisiae* using disc diffusion assay. The leaf extracts showed bactericidal activity against *B. subtilis* only. None of the extracts exhibited any activity against *E. coli* and *S. cerevisiae*. This is because of the presence of different phytoconstitutens in different solvent extracts. Phytochemical analysis of aqueous extract revealed the presence of flavonoids, saponins and carbohydrates but Methanol extract was found to be positive for saponin and glycosides and the same in other species also [8]. Therefore, since medicinal plants have such wonderful antibacterial activities, we have undertaken a study on bactericidal effect of phytoconstituents of medicinal plants present in the campus of Jaipur National University, Jagatpura, Jaipur, Rajasthan with acetone and water as solvents for the extraction of phytoconstituents.

Methods

2.1 Phytoconstituents

Leaves of *Withania sominifera, Azadirachta indica* [9], *Solanum virginianum,* and fruits and rhizome of *Solanum virginianum* and *Alocasia odora* respectively were collected from Jaipur National University campus and dried. Leaves, fruits and rhizome were ground and made fine powder. The powder was stored for extraction. One-gram powder each was dissolved in 50% acetone, 80% acetone and water. The mixture could 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

The dried extracts were dissolved in their respective solvents, diluted to make uniform concentration and stored at -4-degree Celsius fridge for further use. The phytoconstituents were extracted from five different medicinal plant products, table 1 and the extraction was done by maceration method using acetone and water as per the selection of solvents. To analyze the effect of the phytoconstituents, the extraction was done using water, 50% acetone and 80% acetone. To find the dose-wise effect of the phytoconstituents, the phytoconstituents were diluted to get 5mg/ml, 10mg/ml and 20 mg/ml. Table 1 shows all the details of the plants used for phytoconstituents.

Table 1 Name of the plants and the plant parts used in the study

S. No	Name of the plant	Family of plant	Parts used
1	Withania somenifera	Solanaceae	Leaves
2	Azadirachta indica	Meliaceae	Leaves
3	Solanum virginianum	Solanaceae	Leaves
4	Solanum virginianum	Solanaceae	Fruit
5	Alocasia odora	Araceae	Rhizome

2.3 Bacterial Culture medium

NA (nutrient agar) and NB (nutrient broth) both media were used. Nutrient agar was used for the culture and incubation of bacteria. Nutrient broth was used for inhibition study using spectrophotometer after the 2 and 4 hours of growth of bacteria.

For the preparation of 100 ml of Nutrient agar (NA), 1.3 gm of nutrient agar was added in 50 ml of distilled water. Then volume make up was done by adding 50 ml distilled water in it. The nutrient medium was boiled for making agar soluble and autoclaved it. Thereafter 25 ml of NA media was poured in each autoclaved petri plate under laminar flow. Petri plates were left for some time till the media to get solidified. Diseased portion of plants, table 1 was taken from any diseased plant part and cleaned with 80% alcohol and then cleaned with distilled water. Then small diseased plant portions were transferred to the petri plates for inoculation. Sealed Petri plates with paraffin were incubated in an incubator for 2 days at 37°C and then plates were stored at 4°C after the growth. After two days, when the growth of bacteria appeared in the Petri plates, we did gram staining of bacteria for identification. For gram staining, we used primary stain (crystal violet), mordant (Grams iodine), Decolorizer (Ethanol or acetone), counter stain (safranin).

2.2 Bacterial cell suspension

The cells were collected from the culture medium and mounted under microscope to identify the bacteria on study. The cells were washed with sterile water and centrifuged twice at 5000 rpm for 18 minutes to remove the unwanted nutrients. Cells were counted using hemo-cytometer for making cell suspension, cells/ml and stored in 20% glycerol at -4 degree Celsius.

Number of cells per ml = average x division factor x 10^4)

The common pathogenic bacteria used are given in the table 2

Table 2 Name of the pathogen

S. No	Name of the Bacteria	Type of Bacteria	Phylum	Class	Name of the host	Name of the disease
1.	*Clostridium* sp.	Gram-positive	Firmicutes	Clostridia	*Ipomoea batatas*	Bacterial soft rot of sweet potato
2.	*Pseudomonas andropogonis*	Gram-negative	Proteobacteria	Gammaproteobacteria	*Tradescantia sp.*	Leaf spot
3.	*Bacillus sp.*	Gram-positive	Firmicutes	Bacilli	*Tecoma stans*	does not cause disease
4.	*Pseudomonas cichorii*	Gram- negative	Proteobacteria	Gammaproteobacteria	*Plumeria pudica*	Bacterial leaf blight

For the study on bactericidal effect of the phytoconstituents

The inhibition study by all pathogens were conducted as follows: seven ml of NB, 200 micro liters of bacterial cells and 200 micro liters of 50% acetone in one test tube, in other test tube 80% acetone and all as above and the third test tube water and all as above and these were taken as controls and with phytoconstitutents instead of solvents alone as experiments. One test was taken with seven ml of NB, 200 micro liters of bacterial cells only for reference to know the growth of microbes. This is for one plant and one pathogen. Like four pathogen and five plant's phytoconstituents were taken in test tubes with well label. After taking absorbance at 595 nm at 0-hours [10], the experiment starts by keeping all the test tubes in an incubator at 37 degree Celsius. The absorbance was taken after every one hour and the results were recorded for calculating the total inhibition of phytoconstituents alone.

4.1 Results

Table 3 shows the 80% and above inhibition of bacteria by the phytoconstituents of medicinal plants at various concentration.

Table 3, 80% and above Inhibition of the selected bacteria by different selected Phytoconstituents

Phytoconstituents		Inhibition of Bacteria			
Solvent concentration for extraction	**Dose-wise concentration of Phytoconstituents**	Inhibition of *Pseudomonas cichorii*	Inhibition of *Bacillus sp.*	Inhibition of *Clostridium sp.*	Inhibition of *Pseudomonas andropogonis*
Leaves (Azadirachta indica)					
50% Acetone as solvent	***5mg/ml**	*	86%	96%	99%
	***10mg/ml**	*	97%	100%	82%
	***20mg/ml**	83%	94%	99%	102%
80% Acetone as solvent	***5mg/ml**	79%	95%	82%	*
	***10mg/ml**	95%	99%	93%	96%
	***20mg/ml**	99%	100%	88%	100%
Leaves (*Withania somenifera*)					
50% Acetone as solvent	***5mg/ml**	*	84%	90%	104%
	***10mg/ml**	*	93%	98%	85%
	***20mg/ml**	92%	94%	100%	111%
80% Acetone as solvent	***5mg/ml**	96%	99%	84%	94%
	***10mg/ml**	99%	100%	91%	99%
	***20mg/ml**	100%	100%	93%	100%
Rhizome (*Alocasia odora*)					
50% Acetone as solvent	***5mg/ml**	*	72%	96%	97%
	***10mg/ml**	82%	86%	100%	*
	***20mg/ml**	88%	90%	100%	80%
80% Acetone as solvent	***5mg/ml**	90%	96%	*	*
	***10mg/ml**	98%	96%	*	89%
	***20mg/ml**	100%	100%	94%	98%
Fruit (*Solanum virginianum)*					
50% Acetone as solvent	***5mg/ml**	84%	86%	97%	98%
	***10mg/ml**	88%	93%	98%	86%
	***20mg/ml**	90%	96%	100%	118%
80% Acetone as solvent	***5mg/ml**	96%	98%	87%	80%
	***10mg/ml**	96%	99%	93%	97%
	***20mg/ml**	100%	100%	99%	100%
Seed (S*olanum virginianum*)					
50% Acetone as solvent	***5mg/ml**	*	94%	*	106%
	***10mg/ml**	*	93%	100%	98%
	***20mg/ml**	*	94%	100%	106%
80% Acetone as solvent	***5mg/ml**	90%	97%	79%	83%
	***10mg/ml**	96%	99%	*	93%
	***20mg/ml**	98%	100%	81%	96%

* under each bacterium indicates the percentage of inhibition below 80%

It is clear from the table 3 that Bacillus sp. can be inhibited by phytoconstituents extracted from all the plants and parts mentioned in the table at all concentrations, 5mg/ml, 10mg/ml and 20mg/ml. Moreover, the table 3 shows that all species of bacteria mentioned in the table 3 are inhibited by 20mg/ml concentration by all selected plants and parts. Furthermore, S*olanum virginianum* fruit of all concentrations of phytoconstituents inhibits all the selected bacteria. Therefore, deleting the clear results of the table, a table of doubtful concentration of inhibition of bacteria by phytoconstituents is generated, table 4.

Table 4 Inhibition of bacteria by different Phytoconstituents

Phytoconstituents	**Inhibition of Bacteria**			
Solvent concentration for extraction	**Dose-wise concentration of Phytoconstituents**	Inhibition of *Pseudomonas cichorii*	Inhibition of *Clostridium sp.*	Inhibition of *Pseudomonas andropogonis*
Leaves (Azadirachta indica)				
50% Acetone as solvent	***5mg/ml**	72%	96%	99%
	***10mg/ml**	54%	100%	82%
80% Acetone as solvent	***5mg/ml**	79%	82%	66%
	***10mg/ml**	95%	93%	96%
Leaves (*Withania somenifera*)				
50% Acetone as solvent	***5mg/ml**	76%	90%	104%
	***10mg/ml**	50%	98%	85%
80% Acetone as solvent	***5mg/ml**	96%	84%	94%
	***10mg/ml**	99%	91%	99%
Rhizome (*Alocasia odora*)				
50% Acetone as solvent	***5mg/ml**	74%	96%	97%
	***10mg/ml**	82%	100%	67%
80% Acetone as solvent	***5mg/ml**	90%	70%	58%
	***10mg/ml**	98%	69%	89%
Seed (*Solanum virginianum*)				
50% Acetone as solvent	***5mg/ml**	40%	97%	106%
	***10mg/ml**	50%	100%	98%
80% Acetone as solvent	***5mg/ml**	90%	79%	83%
	***10mg/ml**	96%	75%	93%

The table 4 is filled with all the percentage of inhibition in place of * to complete the calculation and find the significant level to calculate the correct concentration of phytoconstituents for inhibition. Since it is a table of many rows and columns, univariate module for each bacterium was used from the SPSS (Statistical Package for Social Sciences) software.

Table 5 Parameter Estimates for *Pseudomonas cichorii*

Parameter	B	Std. Error	t	Sig.	95% Confidence		Observed Power[a]
					Lower Bound	Upper Bound	
[Phytocons =10mg]	97.500	6.912	14.105	.000	81.560	113.440	1.000
[Phytocons =5mg]	63.000	6.912	9.114	.000	47.060	78.940	1.000
[Plants= Aloca50]	15.000	9.776	1.534	.163	-7.543	37.543	.273
[Plants= Aloca80]	-3.500	9.776	-.358	.730	-26.043	19.043	.062
[Plants= Azadi50]	.000	9.776	.000	1.000	-22.543	22.543	.050
[Plants= Azadi80]	-10.500	9.776	-1.074	.314	-33.043	12.043	.158
[Plants= Solan50]	-18.000	9.776	-1.841	.103	-40.543	4.543	.368
[Plants= Solan80]	-4.500	9.776	-.460	.658	-27.043	18.043	.069
[Plants= Withan50]	$_0$b	.	.	.	.	.	.
[Plants= Withan80]	$_0$b	.	.	.	.	.	.

a. Computed using alpha = .05, b. This parameter is set to zero because it is redundant. Phytocons = Phytoconstituents, Aloca50 = Rhizome of *Alocasia odora* in 50% acetone solvent, Aloca80 = Rhizome of *Alocasia odora in* 80% acetone solvent, Azadi50 = *Azadirachta indica* in 50% acetone solvent, Azadi80 = *Azadirachta indica* in 80% acetone solvent, SolFru50 = Fruit of S*olanum virginianum* in 50% acetone solvent, SolFru80 = Fruit of S*olanum virginianum* in 80% acetone solvent, SolSee50 =Seed of Solanum virginianum in 50% acetone solvent, SolSee80 = Seed of Solanum virginianum in 80% acetone solvent

Table 6 Parameter Estimates for *Clostridium sp.*

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval		Observed Power[a]
					Lower Bound	Upper Bound	
[Phytocons =10mg]	87.500	3.021	28.966	.000	80.534	94.466	1.000
[Phytocons =5mg]	94.000	3.021	31.118	.000	87.034	100.966	1.000
[Plants= Aloca50]	4.000	4.272	.936	.377	-5.851	13.851	.132
[Plants= Aloca80]	-18.000	4.272	-4.213	.003	-27.851	-8.149	.956
[Plants= Azadi50]	4.000	4.272	.936	.377	-5.851	13.851	.132
[Plants= Azadi80]	.000	4.272	.000	1.000	-9.851	9.851	.050
[Plants= Solan50]	4.500	4.272	1.053	.323	-5.351	14.351	.154
[Plants= Solan80]	-10.500	4.272	-2.458	.039	-20.351	-.649	.579
[Plants= Withan50]	$_0$b	.	.	.	.	.	.
[Plants= Withan80]	$_0$b	.	.	.	.	.	.

a. Computed using alpha = .05, b. This parameter is set to zero because it is redundant. Phytocons = Phytoconstituents, Aloca50 = Rhizome of *Alocasia odora* in 50% acetone solvent, Aloca80 = Rhizome of *Alocasia odora in* 80% acetone solvent, Azadi50 = *Azadirachta indica* in 50% acetone solvent, Azadi80 = *Azadirachta indica* in 80% acetone solvent, SolFru50 = Fruit of S*olanum virginianum* in 50% acetone solvent, SolFru80 = Fruit of S*olanum virginianum* in 80% acetone solvent, SolSee50 =Seed of Solanum virginianum in 50% acetone solvent, SolSee80 = Seed of Solanum virginianum in 80% acetone solvent

Table 7 Parameter Estimates for *Pseudomonas andropogonis*

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval		Observed Power[a]
					Lower Bound	Upper Bound	
[Phytocons =10mg]	96.500	10.607	9.098	.000	72.041	120.959	1.000
[Phytocons =5mg]	94.500	10.607	8.910	.000	70.041	118.959	1.000
[Plants= Aloca50]	-12.500	15.000	-.833	.429	-47.090	22.090	.114
[Plants= Aloca80]	-23.000	15.000	-1.533	.164	-57.590	11.590	.272
[Plants= Azadi50]	-4.000	15.000	-.267	.796	-38.590	30.590	.056
[Plants= Azadi80]	-15.500	15.000	-1.033	.332	-50.090	19.090	.150
[Plants= Solan50]	7.500	15.000	.500	.631	-27.090	42.090	.073
[Plants= Solan80]	-8.500	15.000	-.567	.586	-43.090	26.090	.079
[Plants= Withan50]	$_0$b	.	.	.	.	.	.
[Plants= Withan80]	$_0$b	.	.	.	.	.	.

a. Computed using alpha = .05, b. This parameter is set to zero because it is redundant. Phytocons = Phytoconstituents, Aloca50 = Rhizome of *Alocasia odora* in 50% acetone solvent, Aloca80 = Rhizome of *Alocasia odora in* 80% acetone solvent, Azadi50 = *Azadirachta indica* in 50% acetone solvent, Azadi80 = *Azadirachta indica* in 80% acetone solvent, SolFru50 = Fruit of S*olanum virginianum* in 50% acetone solvent, SolFru80 = Fruit of S*olanum virginianum* in 80% acetone solvent, SolSee50 =Seed of Solanum virginianum in 50% acetone solvent, SolSee80 = Seed of Solanum virginianum in 80% acetone solvent

Tables 5-7 show that 5mg/ml and 10mg/ml (P-value 0.000 both and Power 1.00) are both effective in inhibiting all selected bacterial species on study however, the plant and parts vary. *Pseudomonas cichorii* can be inhibited effectively by Seed of S*olanum virginianum* in 50% acetone as solvent (P-value 0.103, lowest and the power 0.368), *Clostridium sp.* can be inhibited by using both, Rhizome of *Alocasia odora* and Seed of S*olanum virginianum*, both in 80% acetone solvent

which gives statistically significant result (P-value 0.003, and the power 0.956 very close to 1 and P-value 0.039 and the power 0.579 respectively) and *Pseudomonas andropogonis* can be inhibited by Rhizome of *Alocasia odora* in 80% acetone as solvent ((P- value 0.164, lowest and the power 0.272).

DISCUSSION

Concentrations of the phytoconstituents, 5mg/ml, 10mg/ml and 20mg/ml are obtained from each part of the medicinal plants to find the minimum concentration of the phytoconstituents required for the inhibition of the bacteria.

Bacillus sp. Can be inhibited by any phytoconstitutents at any concentration

It is evident from the table 3 that *Bacillus sp*. are inhibited [11] by all concentrations of phytoconstituents [12] however, inhibition of other bacteria are not clear from the table 3 and hence significant level of inhibition was found by using SPSS software.

High level inhibition at 20mg/ml concentration of phytoconstituents

Phytoconstituents of concentration 20mg/ml of 50% and 80% acetone as solvent for extraction was overdose because all the bacterial species were inhibited by this concentration of phytoconstituents [13], table 3 . The analysis using software gives clarity of statistical significant level for using 5mg/ml and 10 mg/ml concentrations of phytoconstituents for the inhibition of the bacteria selected on study (P-value 0.000 for both concentrations of phytoconstituents and Power 1.00, which is very much required). Therefore, minimum concentration for inhibition can be 5mg/ml or 10mg/ml.

Water as solvent for extraction of phytoconstutents is ineffective as bactericidal.

Phytoconstituents extracted using water as a solvent for extraction to inhibit the bacteria were ineffective because the bacteria were not inhibited by this concentration by all parts of the plants on study (less than 80% of inhibition as per the cutoff). The remaining concentration of the solvents, 50% and 80% acetone used in extraction did not give clear cut inhibition of the selected bacteria on study, taken from the table 3. Therefore, SPSS software was used to find the significant level. The better concentration of solvent for extraction can be 80% acetone mostly and 50% acetone for few, obtained from the tables 5-7.

The inhibition of bacteria by Specific part of medicinal plants by specific concentration of solvent varies.

*Pseudomonas cichorii [14]*can be inhibited effectively by seed of S*olanum virginianum* in 50% acetone as solvent, table 5. *Clostridium sp.* can be inhibited by using both, Rhizome of *Alocasia odora* and seed of S*olanum virginianum* both in 80% acetone as solvent, table 6. *Pseudomonas andropogonis* can be inhibited [15]by Rhizome of *Alocasia odora* in 80% acetone as solvent, table7.

This study is different in different species such as 50 mg/ml or 75 mg/ml are required by different medicinal plant's phytoconstiuents [16]

5.1 SUMMARY OF THE STUDY

For the extraction of phytoconstituents from medicinal plants, 80% acetone as solvent for the extraction may be suitable. All bacterial species on study can be inhibited easily by 20mg/ml concentrations of phytoconstituents of *Withania somnifera* leaves, *Azadirachta indica* leaves, S*olanum virginianum* fruit and seeds and *Alocasia odora* rhizome. For a minimum concentration of phytoconstitutents, 5mg/ml or 10 mg/ml is effective for the inhibition. Water as solvent for extraction of phytoconstitutents for bactericidal is ineffective. *Bacillus* sp. can be inhibited easily by all concentrations and by all medicinal plants on study. *Clostridium sp.* can be inhibited by using both, Rhizome of *Alocasia odora* and seed of S*olanum virginianum*, both in 80% acetone as solvent.

5.2 CONCLUTION

The extracted concentration of phytoconstituents (5mg/ml, 10mg/ml and 20mg/ml) of medicinal plants in this study and the concentration of solvent (50% and 80% acetone and water) used for extraction of phytoconstituents for the inhibition of *Pseudomonas sp., Clostridium sp.* and *Bacillus* sp. vary with concentration of the phytoconstituents and concentration of the solvent used for extraction.

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