

RESEARCH ARTICLE

Synergistic response of *Azadirachta spp.* and *Syzygium spp.* on some fungi due to immunomodulators

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Manuscript details:	ABSTRACT
<p>Received: 01 January, 2015 Revised : 11 February, 2015 Accepted: 24 February, 2015 Published : 30 March, 2015</p> <p>Editor: Dr. Arvind Chavhan</p> <p>Cite this article as: Dassharma Kakoli, Bagkar Pratik and Ravnang Pratik (2015) Synergistic response of <i>Azadirachta spp.</i> and <i>Syzygium spp.</i> on some fungi due to Immunomodulators, <i>Int. J. of Life Sciences</i>, 3(1): 85-90.</p> <p>Acknowledgement: Authors gratefully acknowledge Principle of Bhavan's college department of Botany for their valuable support.</p> <p>Copyright: © 2015 Author(s), This is an open access article under the terms of the Creative Commons Attribution-Non-Commercial - No Derivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.</p>	<p>Medicinal herbs act as immunomodulators supporting the body's immunity system to deal with allergens. Since Neem and Clove are great source of traditional medicines, they were taken up as tool for our study to evaluate their lethal effects on three fungi- <i>Aspergillus</i>, <i>Candida</i> and <i>Penicillium spp.</i> using ethanolic and acetone solvent extracts through Disc Diffusion method. It was clearly observed that Neem had better efficacy as compared to Clove since it showed appreciably higher potency in both the extracts. However the results of the comparative potential of Neem in three fungi was found to be most in <i>Penicillium</i> followed by <i>Aspergillus</i> and least in <i>Candida</i>. Another method was carried out using Well Diffusion method where three different ratio concentrations of Clove : Neem (1:2, 1:1, 2:1) were taken using ethanolic and acetone extracts separately and tried on three organisms at individual level. The result noted showed best antifungal response in 1: 1 followed by 1 : 2 and minimum effect by 2 : 1 which hinted the possibility of the enhanced effect of Clove when paired with Neem. The above experiments proved Neem to be a better fungicide and also yielded enhanced effect when in mixture with Clove.</p> <p>The Spectrophotometric readings for the presence of Flavonoids, phenols, and sterols provided us with the information which showed the concentration of phenols to be more than the others which lead us to believe that in all probability phenols is the active principle behind the better efficiency of <i>Azadirachta</i> as compared to Clove as a fungicide and thus can be pursued as a source of alternative medicine.</p> <p>Keywords: Neem, Clove, fungicide, well diffusion method, disc diffusion method, Synergistic.</p>

INTRODUCTION

The chemical constituents contain many biologically active compounds that can be extracted from neem, including alkaloids, Flavonoids, triterpenoids, phenolic compounds, Carotenoids, steroids and ketones, Azadirachtin is actually a mixture of seven isomeric compounds labeled as azadirachtin A-G and azadirachtin E is more effective (Verkerk and Weight, 1993). Other compounds that have a biological activity are salannin, volatile oils, meliantriol and nimbin (National Research Council, 1992).

Ayurveda is complete health care system, medicinal herbs used in it act as immune-modulators this means it controls infections and enhances body's immune system. Antioxidants are the bio molecules which has potential to overcome various diseases in human body. These carry out free radical scavenging activities and act against reacting oxygen species.

Azadirachta indica commonly called as neem is an ever green tree. The major active constituents of the tree are nimbin, nimbidin and nimbinene (National Research Council, 1992; Biswas *et al.*, 2002). The leaves yield quercetin (Flavonoid) and nimboesterol (β -sitosterol) as well as a number of liminoids (Jacobson, 1990). The trunk bark contains nimbin (0.04%), nimbinene (0.001%), tannins (6.0%), while, the stem bark contains tannins (12-16%) and non-tannins (8-11%) (Biswas *et al.*, 2002). The oil extracted from the seeds contains nimboesterol and flavonoids (Biswas *et al.*, 2002). *Syzygium* species (Fam. Myrtaceae) have been reported to possess antibacterial (Shafi *et al.*, 2002) and anti-inflammatory activity (Muruganadan *et al.*, 2001). It was reported that the buds of *Syzygium aromaticum* (L.) Merr. & Perry (clove) were used in folk medicine as diuretic, odontalgic, stomachic, tonicardiac, aromatic condiment properties and condiment with carminative and stimulant activity (Boulos, 1983). The antimicrobial activity of the essential oils from clove and rosemary (*Rosmarinus officinalis* L.) has been tested alone and in combination (Fu *et al.*, 2007). The immature unopened flower bud of *Syzygium aromaticum* commonly called clove showing rust brown in colour is a tropical tree. The clove consists of free Eugenol, Oleanolic acid, gallotannic acid and methyl-n-amyl ketone. Studies of Thoroski *et al.* (1989) showed that Clove is found to active against most pathogenic and non-pathogenic bacteria and fungi.

The phytochemicals, flavonoids, phenols and sterols possess antifungal, antibacterial, antiviral, antipyretic, anti-inflammatory and analgesic effects. The world around is retuning back to the basics of ayurvedic medicines ayurvedic treatment is non invasive and non-toxic, so it can be used safely as an alternative therapy or along with conventional therapy thus increasing research interest in natural antifungal activities observed in plants. Considering all these aspects, the present study aims at detecting certain antioxidants which may be responsible for the antifungal activities of *A. indica* and *S. aromaticum* against fungi *Aspergillus niger*, *Candida albicans* and *Penicillium chrysogenum*.

MATERIALS AND METHODS

Collection of Neem leaves and Clove buds: Fresh green Neem leaves were selected for the experiment were washed thoroughly to remove dirt and immediately crushed in mortar and pestle to make a paste. Dried clove flowers were powdered in mortar and pestle for experiment.

Solvent Systems: Four solvent systems were used for experiments to obtain different types of bio-molecules in extract. Ethanol, Methanol, Acetone and Water were used as solvent in experiment

Media used: P.D.A. (Potato Dextrose Agar) Composition: Water: 1lit, Potato: 200g, Dextrose: 20g, Agar powder: 20g

Preparation of Inoculums for test organism: Three different fungi *Aspergillus niger*, *Candida albicans* and *Penicillium chrysogenum* were taken from pure cultures and inoculated separately on different PDA plates by 'Streak Plate' method for well diffusion method. For disc diffusion method loop full of culture from culture stock was bulk seeded in culture media.

Methods used for analysis:

Diffusion method: Standard disc diffusion method and well diffusion method were used to study effects of extracts on three different fungi.

Preparation of Neem and Clove extracts: Clove buds and fresh leaves of Neem were individually soaked in four different solvents- Acetone, Ethanol, Methanol and Water in two different flasks for 30-40 min. each

and crushed to get a smooth paste which was filtered with muslin cloth. The collected extracts of clove and Neem were concentrated by evaporation under room temperature.

Sterilized whatman paper discs were dipped in antifungal agent 2% ketoconazole (Effective against all three fungi) was taken in ratio 1:2(determined by trial and error method) with distilled water.

Disc diffusion method: Disc diffusion method was used to study effect of extracts individually on selected fungi. Discs (Diameter:10mm) of clove and Neem extracts and one positive control disc were kept in plates streaked with *Aspergillus niger*, *Candida albicans* and *Penicillium chrysogenum* separately and incubated at 37^o C for 48 hrs.

Well diffusion method: Well diffusion method was used to study synergistic effect of Neem and Clove.

Preparation well and inoculation: In this method the agar plates were allowed to set and equidistance wells of 8mm diameter were made with sterile borer. Three fungi were inoculated on different Petri plates using streak-plate method. 100 μ each extract was propelled directly into the wells of the agar plate. Three different ratios of Clove: Neem extracts viz. 1:2, 1:1, 2:1 were used to check synergistic effect as well as their individual performance. The plates were allowed to stand for 1hr. For diffusion of the extract in to the agar and incubated at 37^o C for 48hrs.

Estimation of phenols, Flavonoids and sterols: Concentration of total phenols, total flavonoids and total sterols were estimated from standard methods- 'Folin Ciocalteau method', and 'Aluminium chloride method' and 'Liebermann-Burchard' method respectively.

RESULTS AND DISCUSSION

Chemical Analysis:

Spectrophotometric Estimation of phenols, Flavonoids and sterols shows that the concentration of active principle phenol is more in both extracts as compared to others (Table 3).

Estimation of Flavonoids is carried out by standard aluminium chloride method (Bansod and Mahendra,

2008). Concentration of Flavonoids in ethanolic extracts of Neem is found to be 192.6 μ g/ml. where as it is found to be 217 μ g/ml in Acetone extract on Neem. For clove concentration of Flavonoids is less as compare to Neem; in ethanolic extract it is found to be 183.6 μ g/ml and in acetone extract it is found to be 207.0 μ g/ml.

Estimation of sterols is carried out by Liebermann-Burchard method. It is found to be 27 μ g/ml and 34 μ g/ml in Ethanolic and Acetone extract respectively, while Clove shows 25 μ g/ml and 18 μ g/ml concentration of Sterols in Ethanolic and Acetone extract respectively.

The concentration of phenolic content in both the extracts was determined using spectro-photometric method (Quettier, 2000). Phenolic concentrations in both Neem and Clove are found to be maximum i.e. 375 μ g/ml.

Antifungal Activity:

A. Disc diffusion method:

In disc diffusion method acetone extracts of Neem shown potential inhibition of all three fungi (*A. niger*, *P. chrysogenum* and *C. albicans*) *P. chrysogenum* is eminently affected by Acetone extract of Neem (A.O.I.: 314.2mm²) followed by *A.niger* (A.O.I.: 153.9mm²) and *C. albicans* (A.O.I.: 113.1mm²)

Ethanolic extract of Neem also showed inhibition of all the three fungi to the lesser extent as compare to acetone extracts of Neem. In contrast to acetone extract *A. niger* is inhibited to greater extent (A.O.I.: 98.5mm²). *P. chrysogenum* and *C. albicans* showed similar area of inhibition (A.O.I.: 95mm²). Methanolic and water extract has no visible inhibitory effect on test fungi.

In disc diffusion method for Clove; Acetone extract shown pronounced effect on all the three fungi (A.O.I.:132.7mm²). While Ethanolic, Methanolic and water extracts has no visible inhibitory effect on any fungi

B. Well diffusion method:

In well diffusion method three different solvent ratios of Neem and Clove were used (1:2, 1:1, 2:1). Acetone extract was found to be the most effective as compared to other three solvents.

Table 1: Disc diffusion method for Neem Extract and Clove Extract

Fungi/Extracts	Area of Inhibition (mm ²)							
	Neem Extract				Clove Extract			
	Acetone	Ethanol	Methanol	Water	Acetone	Ethanol	Methanol	Water
<i>A. niger</i>	153.9	98.5	0	0	132.7	98.5	0	0
<i>P. chrysogenum</i>	314.2	95	0	0	132.7	95	0	0
<i>C. albicans</i>	113.1	95	0	0	132.7	95	0	0

Table 2: Well diffusion method for Neem and Clove Extract:

Fungi/Extracts	Area of Inhibition (mm ²)											
	Acetone			Ethanol			Methanol			Water		
	1:2	1:1	2:1	1:2	1:1	2:1	1:2	1:1	2:1	1:2	1:1	2:1
<i>A. niger</i>	452.4	1256.6	78.54	0	78.54	0	0	0	0	0	0	0
<i>P. chrysogenum</i>	581.1	1809.5	78.54	0	78.54	0	0	0	0	0	0	0
<i>C. albicans</i>	514.7	1398.67	78.54	0	78.54	0	0	0	0	0	0	0

Table 3: Estimation of Flavonoids & Sterols

Sample Extracts	Flavonoids:				Sterols			
	Neem		Clove		Neem		Clove	
	O.D.	Concentration	O.D.	Concentration	O.D.	Concentration	O.D.	Concentration
Ethanol	1.07	192.6µg/ml	1.02	183.6µ/ml	3.0	375µg/ml	3.0	375µg/ml
Acetone	1.21	217.8µg/ml	1.15	207.0µg/ml	3.0	3.75µg/ml	3.0	375µg/ml

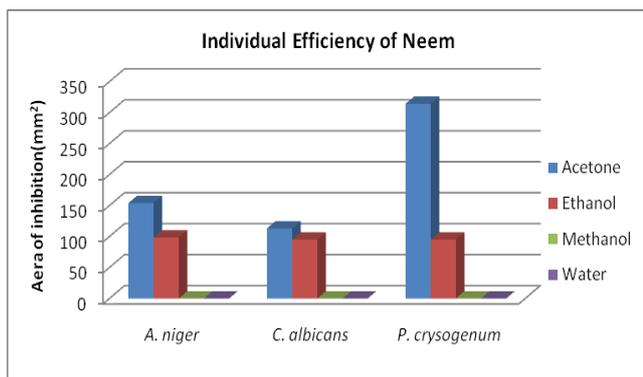


Fig. 1: Inhibition of Neem extracts on three fungi

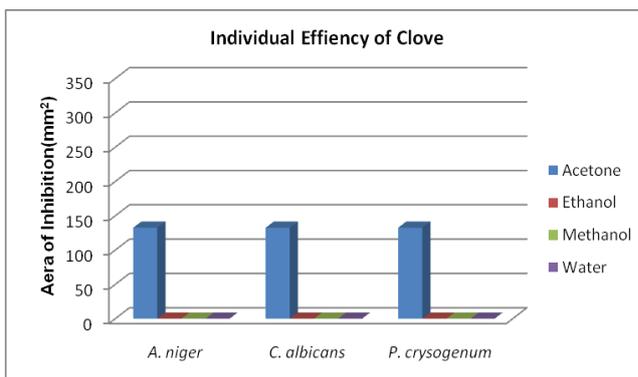


Fig. 2: Inhibition of Clove extracts on three fungi

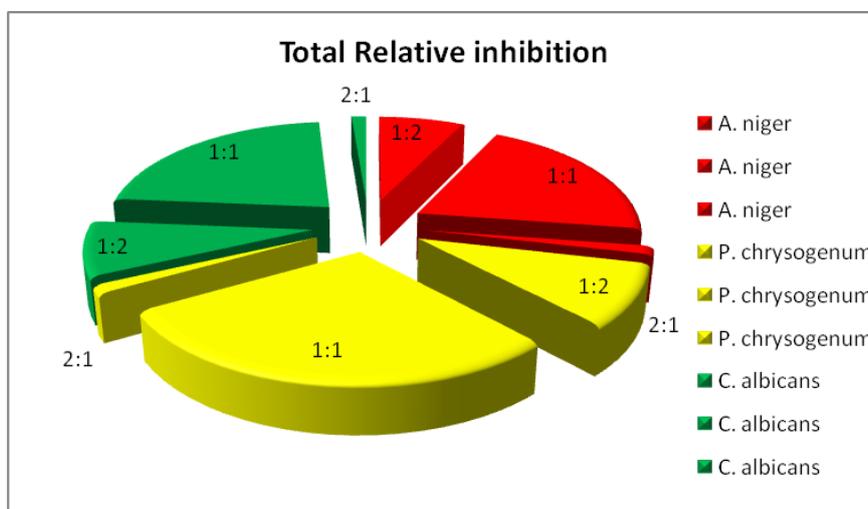


Fig. 3: Relative inhibition of Neem and Clove extracts on three fungi

1:1 ratio of Clove: Neem showed maximum inhibitory effect on all the three fungi i.e. *P. chrysogenum* (A.O.I.:1809.5mm²) followed by *A.niger* (A.O.I.: 1256.6mm²) and *C. albicans* (A.O.I.: 1398.67mm²).

1:2 ratio of Clove: Neem also showed inhibition of all the three fungi but to the lesser extent as that of 1:1 ratio *P. chrysogenum* (A.O.I.:452.4mm²) followed by *A.niger* (A.O.I.: 581.1mm²) and *C. albicans* (A.O.I.: 514.7mm²).

A ratio of 2:1 showed minimum inhibitory response in *P. chrysogenum* (A.O.I. :78.54mm²), *A.niger* (A.O.I.: 78.54mm²) and *C. albicans* (A.O.I.: 78.54mm²) in comparison with test ratios 1:1, 1:2.

Ethanollic extracts also showed notable inhibition of all the three fungi but only with ratio 1:1 *P. chrysogenum* (A.O.I.:78.54mm²) followed by *A.niger* (A.O.I.: 78.54mm²) and *C. albicans* (A.O.I.: 78.54mm²).

Methanol and water extracts have not shown any visible inhibitory effect on any of the test fungi for all three ratios.

From the result of the current study it is clear that Neem has greater antifungal activity as compared to Clove. Effectiveness of Neem oil as a fungicide has earlier been reported by several worker (Lokhande et. al 1998).

The differences in the toxicity of different extracts could be attributed to the presence of the active principles that are extracted by different solvents, which may be influenced by several factors such as age of plant, method of extraction and type of extracting solvent (Nicolls 1969).

Qualitative analysis of both Neem and Clove extracts showed abundance of Phenolic components followed by Flavonoids and Sterols. Acetone extract showed maximum concentration of components/ml of extracts.

From table 1 and 2 it is clear that fungi *P. chrysogenum* is most affected by both the Neem and Clove extract followed by *A. niger* and *C. albicans*.

Ethanollic extracts of Neem also showed notable inhibitory effect on all the test fungi. Madali et al.

(2009) also used Ethanolic extract of Neem for retarding the growth of Aspergillus species.

From the observation of inhibitory effect of extracts of Neem and Clove it is clear that in current studies higher inhibitory principle is released from acetone extract.

In well diffusion method acetone extract of both the Neem and Clove taken individually showed pronounced inhibition. Interestingly 1:1 Acetone extract of Neem and Clove not only showed greater inhibition as a mixture but also showed enhanced inhibitory response as compare to above two plants studied separately; thus indicating synergistic action of Neem and Clove. Thoroski et al (1989) Shown that when Acetone extracts when used in equal amount (1:1) Clove was found to be active against most pathogenic and non-pathogenic fungi.

However 1:2 of Clove: Neem shown comparatively less antifungal response while 2:1 ratio of Clove: Neem showed least antifungal response amongst all test ratios in acetone. Ethanolic extract also showed considerable anti fungal response in test ratio (1:1).

Both aqueous and Methanolic extracts showed no visible antifungal activity on test fungi for any of the test ratio (1:2, 1:1, 2:1).

The further analysis of clove is required since it also shown considerable inhibitory effect against all the three fungi. Bansod and Mahendra (2008) showed that The MIC of *Azadirachta indica* was found to be higher than MIC of clove which is in accordance with our studies. Hence *A. indica* can be considered as alternative of medicine due to its higher potential.

Further investigations are required to isolate and identify the other active principles for both qualitative and quantitative and assessment and their mechanisms of the activity.

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