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Antimicrobial, Synergistic Activity and Antioxidant Studies on Multidrug Resistance Human Pathogen using Crude Extract of *Azadirachta indica* Leaf and *Withania somnifera* Rhizome

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Abstract

We studied effect of *A. indica* and *W. somnifera* crude extracts against the multi-drug resistant strains (MDR) and synergistic activity of *A. indica* and *W. somnifera* crude extracts on MDR strains (Agar well diffusion method). Both aqueous as well as alcoholic extracts of the plant (root as well as leaves) were found to possess strong antibacterial activity against a range of bacteria. Combination exhibited excellent synergy. The aqueous extract and ethanolic extract of *A. indica*and *W. somnifera* exhibited very good synergistic activity against the MDR strain. Extracts were examined for its free radical scavenging activity by DPPH assay. IC50 value was found that 68.75 µg/ml (ethanolic extract) and 74.83 µg/ml (aqueous extract) of *A. indica* and 94.28 µg/ml (ethanolic extract) and 88.79 µg/ml (aqueous extract) of *W. somnifera,* which is significant when compared to solution of Ascorbic acid 50.58 µg/ml. Reducing ability of the crude extracts of *Azadirachta indica* (aqueous and ethanolic extract) and *W. somnifera* (aqueous and ethanolic extract), which is significant when compared to the standard butylatedhydroxy toluene (BHT). Results obtained from this study suggests that *A. indica* and *W. somnifera* (aqueous and ethanolic) extracts may be attractive for the 'drug hunters' as a potential agent for the management of infectious diseases against the human pathogens.

Keywords: *Azadirachta indica*; *Withania somnifera*; Multidrug resistance human pathogen

Introduction

Plants have been a valuable source of natural products for maintaining human health. According to WHO medicinal plants would be the best source to obtain a variety of drugs. We selected *Azadirachta indica* (leaf) and *Withania somnifera* (Rhizome) active constituents of leaf and rhizome on multi-drug resistance (MDR) pathogen.

Review of literature

Yanpallewar, et al. assessed the effect of *Azadirachta indica* (*A. indica*), a plant that has been reported to possess antioxidant, anti-inflammatory and anxiolytic properties, on cerebral reperfusion injury and long term cerebral hypoperfusion [1]. When blood flow to brain region that has undergone critical period of ischemia is re-established, additional injury is to be expected from the reperfusion and study provides an experimental evidence for possible neuro protective potentiality of *A. indica*.

Sureshkumar et al. studied that an ethanolic extract of neem *(Azadirachta indica)* leaf causes cell death of prostate cancer cells (PC-3) by inducing apoptosis as evidenced by a dose-dependent increase in DNA fragmentation and a decrease in cell viability [2]. Western blot studies indicated that treatment with neem extract showed decreased level of Bcl-2, which is anti-apoptotic protein and increased the level of Bax protein. So the neem extract could be potentially effective against prostate cancer treatment.

Owais, et al. evaluated the antibacterial activity of ashwagandha [*Withania somnifera* L. Dunal (Solanaceae; root and leaves)], an Indian traditional medicinal plant against pathogenic bacteria [3]. Both aqueous as well as alcoholic extracts of the plant (root as well as leaves) were found to possess strong antibacterial activity against a range of bacteria, as revealed by *in vitro* agar well diffusion method. The methanolic extract was further subfractionated using various solvents and the butanolic sub-fraction was found to possess maximum inhibitory activity against a spectrum of bacteria including *Salmonella typhimurium*. Moreover, in contrast to the synthetic antibiotic (viz. chloramphenicol), these extracts did not induce lysis on incubation with human erythrocytes, advocating their safety to the living cells. Finally, the antibacterial efficacy of the extracts isolated from plant (both root and leaves) was determined against experimental salmonellosis in Balb/C mice. Oral administration of the aqueous extracts successfully obliterated salmonella infection in Balb/C mice as revealed by increased survival rate as well as less bacterial load in various vital organs of the treated animals.

Boeke, et al. reviewed that the neem tree, *Azadirachta indica*, provides many useful compounds that are used as pesticides and could be applied to protect stored seeds against insects [4]. However, in addition to possible beneficial health effects, such as blood sugar lowering properties, anti-parasitic, anti-inflammatory, and anti-ulcer and hepatoprotective effects, also toxic effects are described and presented a review of the toxicological data from human and animal studies with oral administration of different neem-based preparations. Safety assessments for the various neem-derived preparations were made and the outcomes were compared to the ingestion of residues on food treated with neem preparations as insecticides. This leads to the conclusion that, if applied with care, use of neem derived pesticides as an insecticide should not be discouraged.

Yanpallewar, et al. assessed the effect of *Azadirachta indica* (*A. indica*), a plant that has been reported to possess antioxidant, anti-inflammatory and anxiolytic properties, on cerebral reperfusion injury

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and long term cerebral hypoperfusion [1]. When blood flow to brain region that has undergone critical period of ischemia is re-established, additional injury is to be expected from the reperfusion and study provides an experimental evidence for possible neuro protective potentiality of *A. indica*.

Rationale

To study Antimicrobial, synergistic activity and antioxidant studies on multidrug resistance Human pathogen using crude extract of *Azadirachta indica* leaf and *Withania somnifera* rhzome.

Objectives

To determine the effect of *A. indica* and *W. somnifera* crude extracts against the multi-drug resistant strains (MDR) (Table 1).

To study the synergistic activity of *A. indica* and *W. somnifera* crude extracts on MDR strains. To evaluate the anti-oxidant activity using DPPH free radical method (Table 2).

Methods

Antimicrobial assay of the plant extracts on NCIM cultures

The agar well diffusion method as adopted earlier [5] was used. Test organism was spread on Mueller-Hinton agar plates. The standard inoculum (NCIM cultures) were evenly spread on the surface of the medium by means of sterile cotton swab, The wells of 6 mm diameter were then punched into the agar medium and filled with 60 µl (25 mg/ml) of plant extract, ethanolic and aqueous extract were dissolved DMSO. In one well DMSO was filled in to show that the zone of inhibition was not due to solvent. And 10 µl of ciprofloxacin (Which is used as standard) was filled in and this plate is kept in refrigerator for 20 minutes for diffusion. The plates were incubated for 24 hours at 37°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism.

Antimicrobial assay using plant extract on multi-drug resistant strains (MDR)

The agar well diffusion method as adopted earlier [5] was used test organism was spread on Mueller-Hinton agar plates. Clinical isolate obtained were standardized and this standardized inoculum is evenly spread on the surface of the medium by means of a sterile cotton swab, then wells of 6 mm diameter were punched into the agar medium and filled with 60 µl (50,75 mg/ml) of *A. indica* and *W. somnifera* extracts, both ethanolic and aqueous extract which were dissolved in DMSO. In one well DMSO was filled in to show that the zone of inhibition is not due to solvent. Plate was kept in refrigerator for 20 minutes for diffusion. The plates were incubated for 24 hours at 37°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism (Figure 1).

Synergistic study using *A. Indica* and *W. Somnifera* on MD strains

Synergistic interaction with crude plant extracts of *A. indica* and *W. somnifera* (both ethanolic and aqueous extracts) dissolved in DMSO was studied by agar well diffusion method. For determining the synergistic effects of plant extracts, the wells were punched at a predetermined distances after inoculation of standardized clinical isolates. The wells were loaded with 60 µl (50 mg/ml) of both the plant extracts. The interaction of both the ethanolic extracts and aqueous extracts studied separately. Sixty micro liter of DMSO was loaded and the plates were then incubated at 37˚C, for 18-24 h. Enlargement of inhibition zones indicates a positive interaction (synergism) (Figure 2).

Antioxidant Studies

Free radical scavenging activity of the extracts by DPPH assay

Sample stock solutions (1.0 mg/ml) were diluted to final concentrations of 125,100, 75, 50, 25 µg/ml, in ethanol for *A. indica* crude extracts and 150, 125, 100, 75, 50 µg/ml in ethanol for *W. somnifera* extracts. One ml of a 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solutions of different concentrations, and allowed to react at room temperature. After 30 min, the absorbance values were measured at 518 nm and converted into the percentage antioxidant activity (AA) using the following formula:

$$AA\% = 100 - \{[(Abssample - Absblank) \times 100] / Abscontrolo\}$$

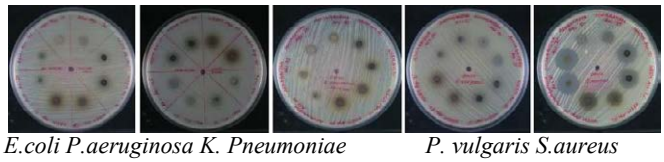
Sl No	Multi-drug resistant strains used	Zone of inhibition (mm)							
		Azadirachta indica				*Withania somnifera*			
		Ethanolic extract conc in mg/ml		Aqueous extract conc in mg/ml		Ethanolic extract conc in mg/ml		Aqueous extract conc mg/ml	
		50	75	50	75	50	75	50	75
1	*S. aureus*	17	25	18	21	21	23	10	13
2	*P. aeruginosa*	10	14	11	15	10	12	11	12
3	*K. pneumonia*	13	15	17	19	11	12	15	17
4	*P. vulgaris*	10	14	10	14	10	12	12	16
5	*E. coli*	14	16	12	14	12	13	10	12

Table 1: Antibacterial activity of crude ethanolic and aqueous extract of *A. indica* (leaf) and *W. somnifera* (rhizome) on multi drug resistant strains.

	Test Multidrug resistant Strains used	Diameter of the inhibition zone in mm											
		A. indica (x) mm		*W. somnifera* (y) mm		Combined Effect (c) mm				% increase on the basis of πr2			
		Ethanolic extract	Aqueous Extract	Ethanolic extract	Aqueous Extract	Ethanolic extract		Aqueous extract		Ethanolic extract (c-x)/x X 100		Aqueous extract (c-y)/y X 100	
						Ai	Ws	Ai	Ws	Ai	Ws	Ai	Ws
1	*S. aureus*	17	12	21	10	22	24	14	13	67.4	30.6	36.2	69.3
2	*E. coli*	14	12	12	10	18	15	15	12	65.3	55.9	56.4	44

KEY: *Ai*: *Azadirachta indica*, *Ws*: *Withania somnifera*. Mean surface area of the inhibition zone (mm^2) was calculated as πr^2 on the basis of their mean diameter (2r) and % increase was calculated based on the formula shown in the table. The zones of inhibition formed singly with respect to x and y and those formed multiply against the same compound were larger in size with respect to MDR strains.

Table 2: Synergistic interaction study using *A. indica* and *W. somnifera* crude extracts on MDR strains.



E.coli P.aeruginosa K. Pneumoniae *P. vulgaris S.aureus*

Figure 1: Antibacterial activity of crude ethanolic and aqueous extract of *A.indica* (leaf) and *W.somnifera* (rhizome) on multi drug resistant strains.

S. aureus *b. E.coli*

Figure 2: Synergistic interaction study using *A.indca* and *W.somnifera* crude extracts on Mdr strains.

Sl no	Sample	Concentration(µg/ml)	% inhibition	*IC50*(µg/ml)
1	Ascorbic acid	20	18.30 ± 0.38	
		40	42.92 ± 0.68	50.58
		60	57.72 ± 0.09	±0.149
		80	84.05 ± 0.08	
		100	90.80 ± 0.4	
2	AIEE	25	27.97 ± 1.06	
		50	38.15 ± 0.52	
		75	52.06 ± 0.04	68.75
		100	67.26 ± 0.47	±0.52
		125	80.59 ± 0.49	
3	AIAE	25	24.88 ± 0.34	
		50	38.82 ± 0.18	
		75	47.14 ± 0.59	74.83
		100	63.48 ± 0.29	±0.574
		125	76.02 ± 0.30	
4	WSEE	50	21.07 ± 0.70	
		75	37.17 ± 0.24	
		100	54.26 ± 0.42	94.28
		125	69.73 ± 0.24	±0.44
		150	86.49 ± 0.19	
5	WSAE	50	28.04 ± 0.50	
		75	42.32 ± 0.05	
		100	56.33 ± 0.38	88.79
		125	70.41 ± 0.41	±0.56
		150	84.48 ± 0.33	

Table 3: Free radical scavenging activity of the extracts by DPPH assay Free radical scavenging activity of AIEE, AIAE, WSEE and WSAE by DPPH method BHT: Butyl hydroxyl toluene; AIEE: *Azadirachta indica* ethanolic extract; AIAE: *Azadirachta indica* aqueous extract; WSEE: *Withania somnifera* ethanolic extract; WSEE: *Withania somnifera* aqueous extract.

Ethanol (1.0 ml) plus plant extract solution (2.5 ml) were used as a blank. DPPH solution (1.0 ml; 0.3 mM) plus ethanol (2.5 ml) was used as a negative control. The positive controls were those using the standard solutions.

The EC50 values were calculated by linear regression if plots where the abscissa represented the concentration of tested plant extracts and the ordinate the average percentage of antioxidant activity from three separate tests (Table 3).

Statistical Analysis

The experiments were done in triplicate. The results are given as mean ± standard deviation (SD). Student's t-test was used for comparison between two means and a one-way analysis of variance (ANOVA) was used for comparison of more than two means. A difference was considered statistically significant when $p \leq 0.05$ assayed. EC50 values obtained from regression lines showed a good coefficient of determination ($r^2 \geq 0.80$) and statistical treatment (ANOVA) of data from the three separate tests shows that all the experiments made for each extract assayed are statistically equivalent ($p<0.05$) 60 µl of extracts were filled in the well. The zone of inhibition was measured by Hi-media zone measuring scale.

Conclusion

The results indicate that the plant extracts have a strong antimicrobial activity. Synergistic study on these two extracts revealed encouraging results in terms of combined zone inhibition and suggests that extracts of *A. indica* and *W. somnifera* can be used as a potential agent for the management of infectious diseases.

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