IN VITRO ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF AZADIRACHTA INDICA, AEGLE MARMELEOS, OCIMUM SANCTUM AND WITHANIA SOMNIFERA EXTRACTS

Rajeev Nema*, Sarita khare

ABSTRACT: This article has brought out and highlighted some Medicinal plants that have therapeutic potential due to the presence of natural antioxidants functioning as reducing agents, free radical scavengers and quenchers of singlet oxygen. Some of the plants selected for the present study, include: Withania somnifera (ASHWANGANDHA) Part Used Leaves, Aegle marmeleos (BEL) Part Used Leaves, Azadirachta indica (NEEM) Part Used Leaves, Ocimum sanctum (TULASI) Part Used Leaves, The goal of present research paper is to determine the use of certain kinds of Medicinal plants antimicrobial activity on Staphylococcus aureus, Bacillus coagulans and one Gram-negative–Escherichia coli, human pathogenic bacteria; and three fungal strains–Aspergillus niger, Tricoderma viride and Fusarium oxysporum. The antibacterial and antifungal activities of extracts (40, 60, 80,100 %) Zone of inhibition of the extracts were compared with that of standards- Streptomycin for antibacterial activity and Griseofulvin for antifungal activity. The results showed remarkable inhibition of the bacterial and fungal growth against the tested organisms.

Key Words: *In vitro Antibacterial, Antifungal, Aegle Marmeleos, Azadirachta Indica, Ocimum Sanctum and Withania Somnifera*

INTRODUCTION

Plants are potential source of chemical constituents with enormous propensity, which synthesize a variety of structurally diverse bioactive compounds [1,2]. Medicinal plants can reduce or minimize the toxic side effect of antibiotics by reinforcing their antimicrobial action [3]. Some of the plants, selected for the present study, include:

Azadirachta indica (Neem)

Known as Neem, is abundant in India All parts of the neem tree can be used such as the fruit, seeds, bark, oil, roots, and leaves and is effective because of its antioxidant levels [4]. Its ingredients have been proven fact on antimicrobial action. Researchers are still going on to evaluate the effects of neem as an alternative treatment that can be taken along side conventional treatment [5].

O. Sanctum (Tulsi)

Tulsi is taken as the most holy plant in India. The use of O. sanctum (Tulsi) as an aromatic plant has been well documented in Ayurveda [6]. Several recent investigations using these extracts have indicated that O. sanctum poses significant anti-inflammatory antioxidant, and immuno-modulatory antibacterial, antioxidant, antiulceric, antimalarial, antidiabetic, antiinflammatory, antilipidemic, anticancer and immunomodulatory properties [7].

W. Somnifera (Ashwagandha)

It is evident from scientific literature that Ashwagandha is rich in foods and has anti-oxidants to play an important role in the prevention of numerous diseases [8]. It had immense medicinal microbial effect. Some of Studies on W. somnifera suggest that it reduces and enhances the effectiveness of conventional therapy with low side effects [9].

A. Marmelos (BEL)

All parts of A. marmelos are medicinally useful like, leaves, fruit pulp, flower, stem bark, root bark. A. marmelos shows a broad spectrum of anti-bacterial, antifungal, anti-inflammatory, antinociceptive and antipyretic activitiy [10,11]. A. marmelos is also widely used in the treatment of hepatitis in folk medicine.

Materials and methods

Plant material collection and Preparation of crude extract:

Plants material Withania somnifera (ASHWANGANDHA) Part Used Leaves, Aegle marmeleos (BEL) Part Used Leaves, Azadirachta indica (NEEM) Part Used Leaves, Ocimum sanctum (TULASI) Part Used Leaves, collected during the month of October from Sanjivani Ayurvedic Nursery Bhopal, These plants were air dried at room temperature. Plants leave of were crushed and extracted with specific solvent hydro alcoholic (1:1) extraction method using soxhlet apparatus. The extract was concentrated under reduced pressure till the mass recorded constant weight at 400C.

Phytochemical analysis:

Withania somnifera, Ocimum Sanctum, Azadirachta indica and Aegle marmeleos were ubjected to phytochemical tests for the identification of various constituents, such as alkaloid, flavonoids, phenolic compound and tannis, carbohydrate, steroid, glycosides, proteins, inorganic acid, oxalic acid, amino acid [12]. Phytochemical screening was performed using standard procedures [13].

Separations analysis:

TLC:

TLC was performed on aluminum plates pre-coated with silica gel 60 (0.063-0.200 mm mesh). Several solvents used were assessed for their ability to fractionate the chemical constituents in the starting material. The mobile phase consisted of solvents (To facilitate mobile phase migration, blotting paper (Whatman 3mm) were allowed to soak and equilibrate for 20 to 30 minutes previous to TLC runs. Detection was carried out by UV light at 254 and 365 nm, and with iodine crystals.

Column chromatography:

After phytochemical analysis bioactive compounds present in extract were separated out by column chromatography in a proper solvent system. Column chromatography was performed on a classic 20 cm long × 2 cm diameter glass column packed with 50 g Silica gel of 60-120 mesh size as stationary phase

All India Institute of Medical Science (AIIMS) Bhopal (Madhya Pradesh), India

Correspondence: Dr. Rajeev Nema, DHR Young Scientist
Department of Biochemistry All India Institute of Medical Science (AIIMS) Bhopal (Madhya Pradesh), India, Tel : +91-9993425698 E-mail: rajeevnema07@gmail.com

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and crude drug were further subjected to column chromatography [CC] and eluted with specific solvent to obtain pure compounds. Silica gel for column chromatography was used as stationary phase. The flow rate used was 5 ml/min. Three and four elutes for each solvent were taken.

Flavonoid Characterization:

FTIR analysis:

FT-IR analysis of the extracts for the detection of functional groups associated was done. The FT-IR spectrum of the plants extracts recorded the number of peaks method were mentioned [14].

Quantitative analysis:

The extract obtained from plant of Withania Somnifera, Terminalia Arjuna, Aegle Marmeleos, Azadirachta Indica, and Ocimum Sanctum were further analysed to estimate the presence of total phenolic and flavonoid contents by various standard chemical and analytical procedures[13].

Total Phenolic Content (TPC):

The total phenolic content was determined by using calibration curve (5 to 10µg/ml).Four readings were taken for each solution for checking the reproducibility and to get accurate result. The intensity of the solution is proportional to the amount of tannins and can be estimated against standard tannic acid, the total phenolic content, expressed as mg tannic acid equivalents per 100 g dry weight of sample [15].The total phenolic content was measured by Folin-Ciocalteu reagent assay.

Total Flavonoid Content (TFC):

Total flavonoid contents were measured by Aluminum chloride colorimetric assay. Hydroalcoholic extracts that has been adjusted to come under the linearity range and different dilution of standard solution of Quercetin (10-100µg/ml) were added to 3ml of water. To the above mixture, 0.1ml of 5% C4H4O6KNa.4H2O (Potassium Sodium L-(+) - Tartrate Tetrahydrate) was added. After 5 minutes, 0.1ml of 10% AlCl3 was added and the total volume was made up to 3 ml with distilled water. It was left at room temperature for 30 min after which the absorbance of the reaction mixture was measured at 430nm [16].

In vitro Antioxidant Assay:

Antioxidant activity of crude extracts was analyzed by standard chemical methods as follows:-

Free radical scavenging activity:

DPPH (2,2 – Diphenyl 1- Picryl Hydrazyl)- The free radical scavenging activity of aqueous and ethanolic extracts and the standard L-Ascorbic Acid (Vitamin C) was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. Here, 0.1mM solution of DPPH in alcohol was prepared and was protected from light influence by maintaining the dark condition and was kept folded in aluminum foil and 3ml of this solution was added to 1ml various conc.(10 µg/ml) of extracts or standard solution of (10 µg/ml). Absorbance was taken after 30min at 550nm. The percentage inhibition activity was calculated from [(A0-A1)/A0] x 100, where A0 is the absorbance of the control and A1 is the absorbance of extract/standard taken as Ascorbic acid.

Nitric oxide scavenging activity:

Nitric oxide radical inhibition was estimated by the use of Griess Illosvory reaction. In this investigation, Griess Illosvory reagent was generally modified by using Napthyl ethylene diamine dihydrochloride (0.1%w/v) instead of using 1-napthylamine (5%) 324. The reaction mixture (3ml) containing 2ml of 10 mM sodium nitroprusside, 0.5ml saline phosphate buffer and 0.5ml of standard solution or aqueous and ethanolic extract of (10µg/ml) were incubated at 25°C for 150min. After incubation, 0.5ml of the reaction mixture was mixed with 1ml Sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5min for the completion of the reaction of diazotization. Further 1ml of the Napthyl ethylene diamine dihydrochloride was added, mixed and was allowed to stand for 30min at 25°C. The concentration of nitrite was assayed at 550nm and was calculated with the control absorbance of the standard nitrite solution (without extracts or standards, but the same condition should be followed).Here the blank was taken as the distilled water and made up solvents and the Ascorbic acid and Quercetin (10 -50 µg/ml) was taken as standard. The percentage inhibition was calculated using the formula:

$$\% \text{ Scavenging Activity} = \frac{\text{A control - A test or A Standard}}{\text{A control}} * 100$$

Where, A control = absorbance of control

A test or A Standard = absorbance of test or std

In-Vitro Scavengers of Nitric Oxide compete with oxygen leading to reduced production of Nitric Oxide [17]. Nitric oxide is generated from sodium nitroprusside (SNP) and was measured by the Griess reagent. SNP in aqueous solution at physiological pH spontaneously generates Nitric Oxide, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess Reagent. The activity is expressed as effective I

Antimicrobial analysis:

Disc diffusion method for antimicrobial activity

Test Microorganisms and Growth Media The following microorganisms Staphylococcus aureus (MTCC 3160), Bacillus coagulans (MTCC 5856) and one Gram-negative—Escherichia coli (MTCC 443), human pathogenic bacteria; and three fungal strains—Aspergillus niger (MTCC), Tricoderma viride (MTCC 800) and Fusarium oxysporum (MTCC 284) were chosen based on their clinical and pharmacological importance.[28] The bacterial strains obtained from Institute of Microbial Technology, Chandigarh, were used for evaluating antimicrobial activity.

In order to detect potential antimicrobial activity in the plant extracts, paper discs (diameter 12 mm) were soaked in an extract solution containing different concentration (40, 60, 80, 100 %). All plates were then incubated at 370C for 24 hr and the zones of inhibition were subsequently measured in mm[18].

Result

Phytochemical Analysis

Some of the parameters which were considered for the study of phytochemical analysis Flavonoids, and Test for tannic and phenolic Phytochemical screening of 4 selected medicinal plants is mentioned in Table No 1.

medicinal plants is mentioned in Table No 1.

S.No.	Test	*Ocimum sanctum* Hydro alcoholic (1:1)	*Withania somnifera* Hydro alcoholic (1:1)	*Aegle marmeleos* Hydro alcoholic (1:1)	*Azadirachta indica* Hydro alcoholic (1:1)
1	Test for falvonoids	+ve	+ve	+ve	+ve
2	Test for tannic and phenolic compound 5% fecl3 Lead acetate Dil. Potassium	 -ve +ve +ve	 -ve +++ve +ve	 -ve +++ve +ve	 -ve ++ve +ve

Qualitative analysis

TLC Analysis

After phytochemical analysis bioactive compounds present in extract was separate out by TLC RF value were identify of all selected plant which is 0.43, 0.50, 0.56, 0.63 and 0.6.(Withania somnifera, Aegle marmeleos, Azadirachta indica, and Ocimum), respectively.

Quantitative analysis

Total polyphenols content of the extracts was found to be .5 µg/g, 120 µg/g, 66 µg/g and 36 µg/g Withania somnifera,, Azadirachta indica, Aegle

marmeleos, and Ocimum sanctum respectively. Total flavonoid content of the extracts was found to be 4.35 µg/ml, 4.78 µg/ml, 4.77 µg/ml and 5.62 µg/ml. Withania somnifera,, Azadirachta indica, Aegle marmeleos, and Ocimum sanctum respectively. Equivalents per dry weight of sample. DPPH Antioxidant IC50 values of the extracts was found to be 44.61 µg/ml, 51.57 µg/ml, 40.0 µg/ml, 74.54 µg/ml and 91.52 µg/ml, Withania somnifera,, Azadirachta indica, Aegle marmeleos, and Ocimum sanctum respectively. Nitric oxide Antioxidant IC50 values of the extracts was found to be 237.86µg/ml, 98.92 µg/ml, 17.18µg/ml,52.91 µg/ml and 312.41 µg/ml Withania somnifera,, Azadirachta indica, Aegle marmeleos, and Ocimum sanctum respectively.

Antimicrobial analysis

Antibacterial and anti fungal activity of Azadirachta indica, Aegle marmeleos, Ocimum sanctum and Withania somnifera

ANTIBACTERIAL ACTIVITY OF COMPOUNDS

*The zone of inhibition (mm) taken as average of four determination in four different direction.

	Diameter of Zone of inhibition (mm)*against														
	(+) *Bacillus coagulans*					(+) *Staphylococcus aureus*					(-) *Escherichia coli*				
	Concentration of compound					Concentration of compound					Concentration of compound				
	100 %	80 %	60 %	40 %	SD	100 %	80 %	60 %	40 %	SD	100 %	80 %	60 %	40 %	SD
A.I	18.2	15.3	14.9	12.7	2.26	18.0	16.5	11.5	10.7	3.61	14.5	12.0	9.7	9.3	2.39
A.M	18.2	16.2	13.6	9.5	3.75	19.3	16.6	12.0	9.5	4.42	14.5	11.3	9.5	6.2	3.46
O.S	20.0	18.5	16.2	14.5	2.43	17.3	14.2	13.0	10.5	2.82	16.5	13.5	10.5	8.3	3.57
W.S	16.2	11.3	10.5	7.5	3.6	18.3	14.5	11.5	8.5	4.19	17.5	13.0	11.5	7.2	4.24
**Std	21.2	20.0	19.5	18.0	1.32	20.0	19.1	18.3	17.9	0.92	18.3	17.0	15.6	14.0	1.84

**Streptomycin used as standard antibacterial agent.

ANTIFUNGAL ACTIVITY OF COMPOUNDS

*The zone of inhibition (mm) taken as average of four determination in four different direction.

	Aspergillus niger					*Tricoderma viride*					*Fusarium oxysporum*				
	Concentration of compound				SD	Concentration of compound				SD	Concentration of compound				SD
	100 %	80 %	60 %	40 %		100 %	80 %	60 %	40 %		100 %	80 %	60 %	40 %	
A.I	15.6	13.9	11.0	9.9	2.61	18.6	16.5	14.0	10.5	3.48	16.0	15.9	11.6	10.8	2.76
A.M	11.5	10.2	6.2	5.0	3.11	3.5	13.0	8.8	6.5	4	13.4	12.5	10.6	7.3	2.7
O.S	14.5	12.5	10.6	8.5	2.56	16.5	13.5	10.8	8.5	3.45	17.5	15.2	16.5	13.3	1.81
W.S	15.5	11.8	7.2	6.0	4.36	13.5	10.0	7.6	5.5	3.43	17.4	15.3	12.1	8.0	4.0
**Std.	16.8	15.2	14.0	12.8	1.7	23.4	22.0	20.9	10.5	5.88	20.1	18.7	17.0	15.3	2.0

**Griseofulvin used as standard antifungal agent.

Statistical Analysis

The results of the inhibition percentage of growth of the three bacteria and three fungi as affected by the four concentrations (40%, 60%, 80%, 100%) of the Azadirachta indica, Aegle marmeleos, Ocimum sanctum and Withania somnifera extracts were statistically analyzed using One way analysis of variance (ANOVA) using Graph-Pad. The results showed significant association of phytochemicals on inhibition of test bacteria and fungi with significant p value p< .05, except For Tricoderma viride No Association Was Found P>.O5.

Conclusion

Some Medicinal plants have therapeutic potential due to the occurrence of natural antioxidants. Plant material Withania somnifera (ASHWANGANDHA) Part Used Leaves, Aegle marmeleos (BEL) Part Used Leaves, Azadirachta indica (NEEM) Part Used Leaves, Ocimum sanctum (TULASI) Part Used Leaves, More research can be done to investigate the unknown and unexplored potential of these plants. Further structural analysis of isolated Flavonoid compounds could be carried out by using different analytical methods such as NMR and Mass spectrometer analysis and which may prove useful for mankind.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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