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Research Article

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In vitro antioxidant, anti-inflammatory, and antimicrobial activity of hydro-alcoholic extract of roots of *Withania somnifera*

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ABSTRACT

Withania somnifera an Indian traditional medicinal herb, is known to possess antistress, antioxidant, analgesic, anti-inflammatory, cardioprotective, adaptogenic, antispasmodic, immunomodulatory and immunostimulant activities. Its other activities include anti-inflammatory, immunoregulatory, anti-tumor, anti-angiogenic, anti-invasive and chemopreventive effects. We investigated the antioxidant, anti-inflammatory and anti-microbial activities of hydro-alcoholic extract of roots of Withania somnifera and tried to relate the role of antioxidant activity in promoting anti-inflammatory activity. 1,1-Diphenyl-2-picrylhydrazyl and nitric oxide radical scavenging assays were used to evaluate antioxidant potential whereas anti-inflammatory activity was assessed by HRBC membrane stabilization method and albumin denaturation assay. Antibacterial activity against test organisms E. coli and S. aurens was evaluated and for antifungal activity, extract was tested against Aspergillus fumigatus. Our results suggest that WS extract possesses potent antioxidant activity, significant anti-inflammatory activity and noteworthy anti-microbial activity against E. coli and S. aureous. Unfortunately, the extract was found to be inactive against Aspergillus fumigatus.

Keywords: *Withania somnifera*, Hydroalcoholic, Antibacterial, Anti-inflammatory, Antioxidant.

INTRODUCTION

Reactive oxygen species like superoxide anion radicals (O2-), hydroxyl radicals (OH.) and non-free-radical species such as H_2O_2 and singled oxygen are involved in promoting cellular injury and ageing. ROS are known to damage macromolecules like proteins, DNA and lipids whereas antioxidants suppress their action by scavenging them [1-2]. ROS are also responsible in development of systemic inflammatory response syndrome and then they activate nuclear factors thus inducing the synthesis of cytokines. Later, inflammatory mediators and adhesion molecules are also formed. Free radicals react with different cell components at site of inflammation thus leading to loss of function and cell death [3].

Withania somnifera (WS) is an Indian traditional herb, used for treatment of nervous exhaustion, memory related conditions, insomnia, tiredness, potency issues, skin problems and coughing. It is known to be a potent aphrodisiac rejuvenate. In Sanskrit, it is known as Ashwagandha. It grows in dry areas in India, on the Himalayas, Baluchistan, Sri Lanka and in the Mediterranean area (Sicily and Sardinia) [4-5]. WS is known to possess antistress, antioxidant, analgesic, anti-inflammatory, cardioprotective, adaptogenic, antispasmodic, immunomodulatory and immunostimulant activities [6]. The present study was conducted to reveal *in vitro* antioxidant, anti-inflammatory and antimicrobial activities of hydroalcoholic extract of WS. Antioxidant assay was conducted by DPPH and nitric oxide scavenging assay. For *in vitro* anti-inflammatory activity, Human red blood cell (HRBC) and albumin denaturation assay assays were performed. Anti-microbial activities were evaluated against gram positive and gram negative strain i.e. E. coli and S. aurens respectively whereas antifungal was assessed against *Aspergillus fumigatus.*

EXPERIMENTAL SECTION

Dried roots of WS were purchased from Natural Remedies, Bangalore. For extraction, powdered air dried roots of WS were subjected to soxhlet extraction for 36 hours by using petroleum ether for defatting. Defatted material was then subjected to soxhlet extraction using hydroalcoholic mixture of ethanol:water (70:30) for 72 hours. Rota evaporation was performed followed by lyophillization to get extract in dried form.

Phytochemical analysis
The extract was subjected to phytochemical screening to evaluate the presence of chemicals constituents as found in literature survey.

Evaluation of antioxidant assay
1,1-Diphenyl-2-picrylhydrazyl radical scavenging assay [2]
0.5 mL of DPPH was added to 0.5 mL of standard or test fraction solution (2, 4, 8, 16, 32, 64, 128, 256, 512 and 1000 µg/mL) in test tubes. Control test tubes were loaded with 0.5 mL of DMSO and 0.5 mL DPPH. The tubes were incubated at 37^{o}C for 30 minutes, prevented from light exposure and the absorbance of each solution was measured at 517 nm in Ultra-Violet spectroscopy and readings were taken triplicate. Ascorbic acid was used as a standard. The percentage scavenging by test fractions at each concentration was calculated by using the formula:

Scavenging DPPH (%) = [($_{Absorbance}$control −$_{Absorbance}$sample)/$_{Absorbance}$control] × 100

IC_{50} represents the level where 50% of the radicals were scavenged by test samples.

Nitric oxide scavenging assay [2]
Griess reagent was prepared by mixing of solution-1 (1% sulphanilamide in 25% v/v hydrochloric acid) and solution-2 (0.01% naphthyl ethylenediamine) in equal volumes. Solution of sodium nitroprusside (5mM) in standard phosphate buffer (0.025 M, pH-7.4) was prepared. This solution was incubated with different concentrations (2, 4, 8, 16, 32, 64, 128, 256, 512 and 1000 µg/mL) of fractions at 37˚C for 5 h. Control was performed in same way but without the test fraction. After 5 h, 0.05 mL of incubated solution was removed and diluted with 0.5 mL of Griess reagent. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthyl ethylenediamine was read at 546 nm and readings were taken triplicate. Ascorbic acid was used as a standard. The percentage scavenging by test fractions at each concentration was calculated by using the formula:

Scavenging NO (%) = [($_{Absorbance}$control −$_{Absorbance}$sample)/$_{Absorbance}$control] × 100

IC_{50} represents the level where 50% of the radicals were scavenged by test samples.

Anti-inflammatory activity
HRBC membrane stabilization method [7-8]
This method is used to study anti-inflammatory activity. Basically, observations and conclusion for anti-inflammatory activity are made on the basis of stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis. Assay mixture is prepared by mixing 0.5mL of plant extract (500, 1000, 1500, 2000, 2500µg/ml), with 1ml phosphate buffer [pH 7.4, 0.15 M], 2 ml hypo saline (0.36 %), 0.5 ml HRBC suspension (10 % v/v). Diclofenac sodium is used as test drug. The haemoglobin suspension was observed at 560nm in spectrophotometer. Haemolysis produced in the presence of distilled water was taken as 100%. And percentage of HRBC membrane stabilization/protection was calculated using the formula,

Percentage stabilization = 100 - [(optical density of test solution)/ (optical density of control) × 100].

Albumin denaturation assay [9]
0.2% W/V of BSA was dissolved in Tris buffer and pH was adjusted to 6.8. Both extract and standard drug (diclofenac sodium) were diluted to (500, 1000, 1500, 2000, 2500µg/ml). 5ml of 0.2% W/V BSA was transferred to eppendorf tubes containing 50µg/mL of extract/standard. The solution was heated at 72˚C and cooled at room temperature for 15 minutes. The absorbance of solution was read at 660nm in spectrophotometer and percentage inhibition was calculated using formula-

%Inhibition= (Abs control - Abs sample)/Abscontrol×100.

Anti-microbial assay [10-11]
In this study *E. coli* (MTCC-1885) as gram negative bacteria and *S. aureous* (MTCC-1144) as gram positive bacteria were used as a model bacterium to study the anti-bacterial activity of various extract of *Withania somnifera* via both quantitative analysis in liquid Luria Broth (LB) medium and qualitative analysis on solid Luria Broth Agar (LBA) medium. Ampicillin was used as a standard for gram negative bacterial culture and vancomycin was used as a standard for gram positive bacterium. For qualitative antimicrobial analysis, corresponding bacterium are cultured on the LB agar plates and analysed for zone of inhibition. Briefly the bacterial suspension of 100µl was first poured and spreaded onto the agar plates. The plates were kept untouched for 10-15minutes so that bacteria get sufficient time to adhere on the surface of agar plate. Suitable well cutter was used to make the well on the agar plate. All the collected plant extracts were sterilized by the exposure of ultraviolet radiation for 3 hour prior to be seeded into the well. The bacteria were then incubated at 37^{o}C for 24 hours and the bacterial inhibition zone for each extracts on the plate was visually inspected. Calculations for inner and outer diameters along with diameter differences were measured.

Anti-fungal Activity [10,12]
All the extracts were dissolved in deionised water to attain the concentration of 2500 µg/ml. Potato dextrose agar (PDA) medium was prepared and autoclaved at 130^{o}C for at least 25 min Aspergillus (MTCC- 8877) species culture were grown on PDA at 37^{o}C until sporulation occurs, generally for the time period of five days. Approximately 30 ml of the medium was poured into the petri dish and kept untouched until the medium gets solidify to form hard surface which is suitable for streaking. Available cork borer was used to cut the wells, approximately 6 mm in diameter. Fungus was streaked on to the surface of the solidified medium to achieve the uniform culture bed. To the bored wells on the petri plate, 2500 µg/ml of the methanolic extract was seeded along with the standard Fluconazole and the plate was incubated at 37 ± 1^{o}C for 24 hours in available incubator.

Inhibition growth percentage was calculated according to the following formula:

% Inhibition Growth= Growth in control-Growth in treatment/Growth in control × 100

RESULTS AND DISCUSSION

Phytochemical Analysis
Phytochemical screening of WS root extract revealed the presence of alkaloids, saponins, tannins, flavonoids, terpenoids and phenolic compounds as shown in (table 1):

Table-1 Phytochemical screening of *Withania somnifera*

Phytoconstituents	**Hydroalcoholic extract of *Withania somnifera***
Alkaloids	+
Saponins	+
Tannins	+
Flavonoids	+
Phenolic compound	+
Terpenoids	+
Flavone glycosides	-
Carbohydrates	+
Triterpenoids	-

Table-2. Antioxidant activity of *Withania somnifera* as per DPPH radical scavenging and nitric oxide scavenging assay

S.No.	**IC50 (µg/mL)**		
	Assay	Ascorbic acid	Withania somnifera
1.	DPPH radical scavenging activity	15 ± 2.1	352 ± 22
2.	Nitric oxide scavenging activity	17 ± 1.7	378 ± 29

Table-3. Anti-inflammatory activity of *Withania somnifera* as per Inhibition of albumin denaturation assay

S.No.	**Concentration**	**HRBC Stabilizing Assay (% inhibition)**	
	µg/mL	Diclofenac sodium	*Withania somnifera*
1	2500	525	350
2	2000	470	297
3	1500	410	225
4	1000	383	170
5	500	295	110

Antioxidant activity and anti-inflammatory activity

WS extract showed significant antioxidant activity (table-2) and anti-inflammatory activity (table-3, 4).

Table-4. Anti-inflammatory activity of *Withania somnifera* as per HRBC Stabilizing Assay

S.No.	Concentration (µg/mL)	Inhibition of albumin denaturation (% inhibition)	
		Diclofenac sodium	*Withania somnifera*
1	2500	775	615
2	2000	600	485
3	1500	310	210
4	1000	178	95
5	500	75	29

Antibacterial Activity:

Extract was capable of inhibiting the growth of bacterial strains used, as shown in (table 5) and (figure 1)

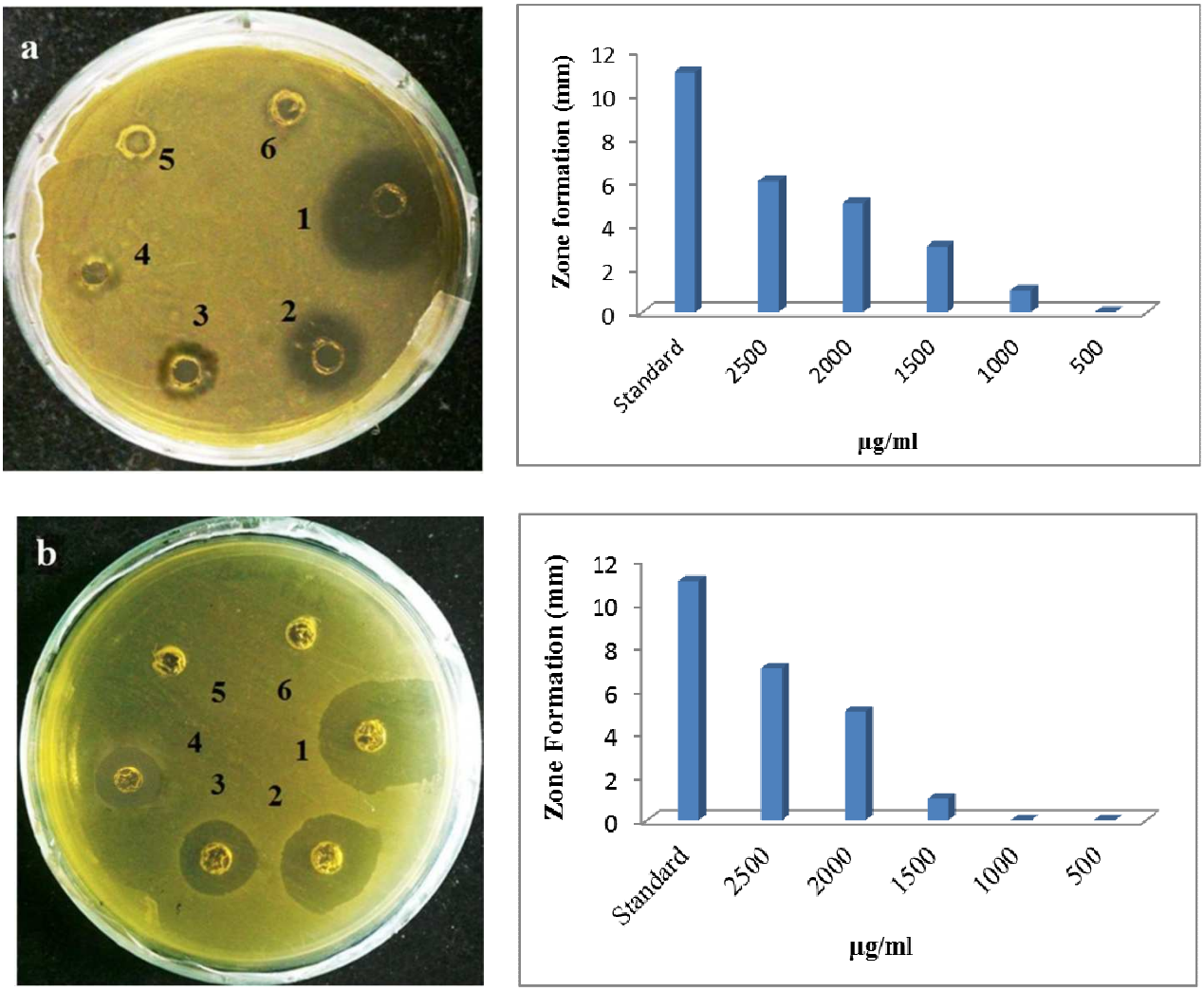

Figure 1. Qualitative growth inhibition assay of bacteria on agar plates with effect on (a) *E.coli* and (b) *S.aureus* after 24 h incubation with various extract of *Withania somnifera*

There is a great rise in use of herbals as medications. About 80% population of developing countries have been estimated to rely on traditional medicines for their primary health care [13]. Prabu et al. assessed the toxicity potential of WS root extract and reported that WS extract is safe to consume [14]. Active constituents present in roots of WS are alkaloids (isopellertierine, anferine), steroidal lactones (withanolides, withaferins), saponins (sitoindoside VII and VIII) and withanoloides with a glucose at carbon 27 (sitonidoside XI and X) [15-16]. Singh et al. evaluated antimicrobial activity and concluded that WS possesses great antimicrobial potential against various test bacterial and fungal strains used [8]. Alcoholic extract of WS leaves possesses potential to enhance

differentiation of glial cells [9]. Roumy et al. reported the antifungal and cytotoxic activity of withanolides [17]. Mainly alkaloids and steroidal lactones commonly i.e. withanolides are responsible for their activities like anti-inflammatory, immunoregulatory, anti-tumor, anti-angiogenic, anti-invasive, and chemo-preventive effects [18]. Glycowithanolides present in WS are also known to possess potent antioxidant activity [19]. Saponins present in WS which have been known to possess potent antioxidant and antimicrobial activity [20-21].

Table 5: Diameter of bacterial inhibition rings for different extract of *Withania somnifera*

	E. coli			***S. aureous***		
Samples µg/mL	**External Diameter (mm)**	**Inner Diameter (mm)**	**Diameter Difference (mm)**	**External Diameter (mm)**	**Inner Diameter (mm)**	**Diameter Difference (mm)**
Standard	19 ± 0.15	06 ± 0.07	13 ± 0.08	18 ± 0.15	07 ± 0.12	11 ± 0.03
2500	12 ± 0.13	06 ± 0.1	06 ± 0.02	14 ± 0.13	07 ± 0.11	09 ± 0.02
2000	10 ±0.04	06 ± 0.05	04 ± 0.99	12 ± 0.4	07 ± 0.4	05 ± 0.83
1500	08 ± 0.1	06 ± 0.1	02	08 ± 0.3	07 ± 0.3	01
1000	07 ± 0.2	06 ± 0.2	01	0	0	0
500	0	0	0	0	0	0

CONCLUSION

Antioxidants can help in reducing the incidences of inflammation. Multiple antioxidants if consumed in diet, normalized ROS induced release of interleukin-6, thus preventing incidences of lipid peroxidation [22]. Multi antioxidants are also known to reduce inflammatory symptoms in inflammatory joint disease, acute and chronic pancreatitis, and adult respiratory distress syndrome [23]. Thus from the present investigation it can be concluded that antioxidant activity may account for anti-inflammatory activity of WS root extract. WS extract showed significant antioxidant and anti-inflammatory activity. Extract was also capable of inhibiting the growth of bacterial strains used. Effect was seen in a concentration manner. Unfortunately, the extract possessed no activity against fungal strain. The extract can be further studied other to explore mechanism and constituent(s) involved in its activity.

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