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PRELIMINARY PHYTOCHEMICAL SCREENING, IN VITRO ANTIOXIDANT AND CYTOTOXIC ACTIVITY OF FIVE DIFFERENT EXTRACTS OF *WITHANIA SOMNIFERA* ROOT

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ABSTRACT

Current investigation aimed to assess the phytochemical screening, free radical scavenging potential and cytotoxic activity of the root extracts of *Withania somnifera* (Ashwagandha). Phytochemical screening revealed the presence of phenol, flavonoid, tannin, saponin, alkaloid, glycoside and carbohydrate. In evaluation, methanol, ethanol, chloroform, pet-ether and n-hexane extract of the root powder was prepared and screened for *in-vitro* antioxidant activities by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity where chloroform, pet-ether and methanol extract of *Withania somnifera* showed noticeable effect in the DPPH scavenging assay. All five extracts were subjected to brine shrimp lethality bioassay for possible cytotoxicity. Concentration dependent increment in percent mortality of Brine Shrimp nauplii produced by the ethanol extract of *Withania somnifera* indicates the presence of cytotoxic principles in these extractives.

Keywords: Phytochemical screening, Withania somnifera, Antioxidant, free radical, DPPH, Brine shrimp lethality bioassay.

INTRODUCTION

Recognition and development of the potent medicinal plant and economic benefits from these plants has attained an increased attention. For over a decade, interest has been revived in the study and use of traditional medicine in different parts of the world. As a result, countries have sought cooperation in identifying and using safe positive components of traditional medicine in their national health systems [1].

There is increasing awareness that many components of traditional medicine are beneficial while others may be harmful, hence the World Health Organization (WHO) encourages and supports countries to identify and provide safe and effective remedies for use in the public and private health services [1]. *Withania somnifera* appears to be a dense pubescent shrub that grows up to a height of 1 miter tall and belongs to the family of solanaceae. It is a popular medicinal plant of Bangladesh and locally known as ashwagandha.

This plant is capable of growing wildly not only in all the drier parts of the subtropical Bangladesh i.e. in nator, savar, and north-western parts of Bangladesh but also in India, Congo, South Africa, Egypt, Morocco, Jordan, Pakistan and Afghanistan. The roots are the main portions of the whole plant as they possess wide number of the therapeutic agents. The crude aqueous extract of the plant contains the phenolics and flavonoids which are said to be the potent antioxidants [2].

Ashwagandha is found to be a major ingredient of various adaptogenic and anti-stress tonics [3]. A methanolic extract of the various parts of *Withania somnifera* had showed a potent anti-inflammatory activity. *Withania somnifera* is found to be a unique plant where a wider range of biological activities has been demonstrated including antagonism with several

inflammatory factors and the immune modulation. *Withania somnifera* have been found to exhibit certain antibacterial, anti-fungal and antitumor properties [4]. The mechanism of action of the pharmacological properties such as the anti-inflammatory, anti-tumor, anti-stress, anti-oxidant, immune-modulatory, hemopoietic, rejuvenating were determined but not fully understood. The objective has been to explore and evaluate the Phytoconstituents, Antioxidant activity to provide a suitable lead and cytotoxicity which may be consumed in future to trigger a new line of investigation, based on the combined approach of both exploitation and exploration.

MATERIALS AND METHODS

Chemicals: 1, 1 diphenyl-2-picrylhydrazyl (DPPH), Vincristine Sulfate was obtained from Sigma Aldrich. Other chemicals, Methanol, Butylated hydroxytoluene (BHT), Concentrated H_2SO_4 (96%) were obtained from (Merck KGaA, Darmstadt, Germany). Ascorbic acid, Ferric Chloride, Picric acid, Lead acetate from Loba Chemie Pvt.Ltd, India. Sodium chloride crystal, Sodium hydroxide pellets, Gelatin, Chloroform, Dimethyl sulfoxide (DMSO), 1-Naphthol from Merck Specialities Private Limited, India. All other reagents and chemicals used were of analytical grade.

Collection of plant sample: Plant sample of *Withania somnifera* was collected from suburb of Dhaka, Bangladesh. After complete cleaning roots were sun dried. The dried roots were then grinded in coarse powder using high capacity grinding machine which was then stored in air-tight container with necessary markings for identification and kept in cool, dark and dry place for the investigation.

Extraction procedure: The powdered plant material (20 gm) was successively extracted in a Soxhlet extractor at elevated temperature using 200 ml of distilled petroleum ether (40-60)°C followed by n-hexane, ethanol, chloroform and methanol. All extracts were filtered individually through filter paper and extracts were collected through rotor evaporator. After drying in desicator, crude extracts were weighed and stored in stock vials and kept in refrigerator (0- 4) °C for farther use.

Phytochemical screening: Preliminary phyto chemical screening was performed to identify the various classes of active chemical constituents such as phenols, flavonoids, tannins, saponins, alkaloids, glycosides and carbohydrates.

DPPH free radical scavenging assay: The free radical scavenging capacity of the extracts was determined using DPPH [5]. Freshly prepared DPPH solution was taken in test tubes and extracts were added followed by serial dilutions (15.625 - 250 µg/ml) to every test tube so that the final volume was 5 ml and after 30 min, the absorbance was read at 517 nm using a spectrophotometer. Ascorbic acid and Butylated hydroxy toluene (BHT) was used as standard. Control sample was prepared containing the same volume without any extract and standard and the absorbance was read at 517 nm using a spectrophotometer. Methanol was served as blank.

Brine shrimp lethality bioassay: Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds [6]. The brine shrimp, *Artemia salina*, was used as a convenient monitor for the screening. The eggs of the brine shrimp, *A. salina*, were collected from an aquarium shop (Dhaka, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution, pH-8.5) for 48 hr to mature shrimp called nauplii.

The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method. The test samples (extract) were prepared by dissolving in DMSO (not more than 50 µl in 5 ml solution) with sea water (3.8% NaCl in water) to attain concentrations 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 µg/ml. In the present study Standard vincristine sulfate was used as positive control. As vincristine is a very cytotoxic alkaloid and it was evaluated at very low concentration (40, 20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078 µg/ml).

Then matured shrimps were applied to each of all experimental vials and control vial. After 24 hours, the vials were inspected using a magnifying glass and the number of surviving nauplii in each vial were counted. The lethal concentrations of plant extract resulting in 50% mortality of the brine shrimp (LC_{50}) from the 24 h counts and the dose-response data were transformed into a straight line by means of a trend line fit linear regression analysis. The LC_{50} was derived from the best-fit line obtained.

RESULTS AND DISCUSSION

Phytochemical screening: Preliminary Phytochemical screening of the crude extracts of root of *Withania somnifera* revealed the presence of different kind of chemical groups that are summarized in table 1.

Methanol extracts of root of *Withania somnifera* contain phenols, Flavonoids, saponins, alkaloids, glycosides and carbohydrate. Ethanol extracts also possess those phytoconstituents except flavonoids and saponins. Pet-ether extract of root of *Withania somnifera* contains Flavonoids, tannins, alkaloids, glycosides, saponins and carbohydrates and n-hexane extract contains carbohydrates and flavonoids. Chloroform extract contains phenols, flavonoids and tannins. Water extract of root of *Withania somnifera* contains tannins, Saponins, alkaloids and carbohydrate.

DPPH free radical scavenging assay: The free radical scavenging activity of different extracts of *Withania somnifera* root was studied by its ability to reduce the DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH, can react with it and thereby bleach the DPPH absorption. DPPH is a purple colour dye having absorption maxima of 517 nm and upon reaction with a hydrogen donor the purple colour fades or disappears due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance. Chloroform, pet-ether and methanol extracts showed maximum activity of 75.37%, 63.61% and 54.16% respectively at 250 µg /ml, where as ascorbic acid and BHT at the same concentration exhibited 96.66% and 92.59 % inhibition respectively.

Five extracts exhibited considerable DPPH free radical scavenging activity as indicated by their IC_{50} values and this has been showed in (Table 2 and Figure 1). IC_{50} Indicate the potency of scavenging activity. Standard ascorbic acid and BHT were found to have an IC_{50} of 5.698 µg/ml and 8.816 µg/ml. In comparison to standard ascorbic acid and BHT, chloroform, pet-ether and methanol extract of *Withania somnifera* root showed IC_{50} of 87.414, 144.998 and 267.818 respectively. Ethanol and n-hexane fraction is seen to have the least free radical scavenging activity.

Brine shrimp lethality bioassay: In the present bioactivity study the five crude extracts and pure compounds showed positive results indicating that the test samples are biologically active. The methanol, ethanol, petroleum ether, n-hexane and chloroform extract of the dried root of *Withania somnifera* were subjected to brine shrimp lethality bioassay following the procedure which has been utilized by Meyer *et al.*, 1982. The lethality of the extractives to brine shrimps was determined and the results are given in Table 3.

The lethal concentration (LC_{50}) of the test samples after 24 hours was obtained by a plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. Vincristine Sulphate (VS) was used as positive control and the LC_{50} was found as 0.323 µg/ml. Compared with the negative control, VS (positive control) gave significant mortality and the LC_{50} values of the different extractives were compared with negative control.

The LC_{50} values of methanol, ethanol, petroleum ether, n-hexane and chloroform were found to be 4.112 µg/ml, 1.938 µg/ml, 2.269, 3.732 µg/ml and 3.450 respectively (Table 3). However, varying degree of lethality of *Artemia salina* was observed with exposure to different dose levels top the test samples ranging from 0.781-400 µg/ml.

The degree of lethality shown by the extractives was found to be directly proportional to the concentration of the extractives ranging from the lowest concentration (0.781 µg/ml) to the highest concentration (400 µg/ml). This concentration dependent increment in percent mortality of Brine Shrimp nauplii produced by the *Withania somnifera* extracts indicates the presence of cytotoxic principles in these extractives. There was no mortality in the negative control groups indicating the test as a valid one and the results obtained are only due to the activity of the test agents.

CONCLUSION

It can be concluded that the extracts of the *Withania somnifera* can be used to design different antimicrobial agents due to its moderate cytotoxic nature. Further work is needed to isolate the secondary metabolites and study of metabolic interchanges in bacterial metabolic pathways when applying this extract. This *in vitro* study demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. The use of these plants in folk medicine suggests that they represent an economic and safe alternative to treat infectious diseases.

Table 1: Result of chemical group test of various extracts of root of *Withania somnifera*

TESTS	EXTRACT					
	Methanol	**Ethanol**	**Pet-ether**	**n-hexane**	**Chloroform**	**Water**
Phenols	+	+	-	-	+	-
Flavonoids	+	-	+	+	+	-
Tannin	-	-	+	-	+	+
Saponin	+	-	+	-	-	+
Alkaloids	+	+	+	-	-	+
Glycosides	+	+	+	-	-	-
Carbohydrate	+	+	+	+	-	+

+++: Present in high concentration, ++: Present in moderate concentration, +: Present in low concentration and -: Absent

Table 2: IC_{50} values of different extracts of *Withania somnifera* in DPPH scavenging assay

Extracts/standard	**IC_{50} µg/ml**
Methanol	267.818
Ethanol	529.484
Pet-ether	144.998
n-hexane	1236.400
Chloroform	87.414
Ascorbic acid	5.698
Butylated hydroxy toluene (BHT)	8.816

Table-3: LC_{50} values of the five extracts of *Withania somnifera* and standard

Test Samples	**Regression line**	**R^2**	**LC_{50} values**
Vincristine	y = 29.79x + 64.62	R^2 = 0.927	0.323
Methanol	y = 29.998x+31.58	R^2 = 0.9489	4.112
Ethanol	y = 23.958x+43.114	R^2 = 0.9731	1.938
n-hexane	y = 25.166x+35.607	R^2 = 0.9088	3.732
Chloroform	y = 23.958x+37.114	R^2 = 0.9731	3.450
Pet-ether	y = 23.555x+41.616	R^2 = 0.9239	2.269

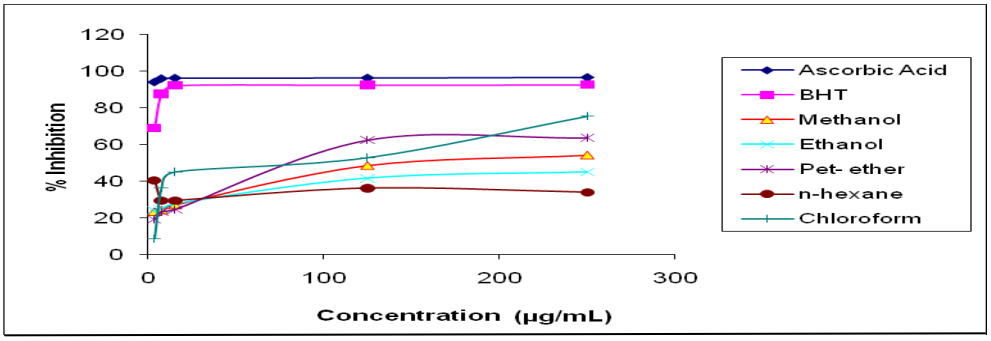

Figure 1: Comparative DPPH radical scavenging activity of *Withania somnifera* root extract, Ascorbic acid and Butylated hydroxy toluene (BHT)

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