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Research Article

Phytochemical and Microbiological Evaluation of a Local Medicinal Plant *Bacopa monnieri* (l.) Penn.

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ABSTRACT

This research work was carried out to investigate the phytochemical constituents as well as the pharmacological prooperties of 'Bacopa monnieri'. The project work reveals that the plant has antibacterial and antimutagenic activities. Thus an attempt was made to reinvestigate aforementioned antimicrobial as well as cytotoxic properties for the bark constituents. The following aspects of Bacopa monnieri (L.) Penn., (root bark and stem bark) have been intended to be accomplished in this research project: Review of the chemical constituents and biological activities of Bacopa monnieri (L.) Penn and the successive isolation of the dried bark (root and stem) with alcohol. The powdered plant was successively extracted with ether solvent. The Phytochemical screening of the extracts showed the presence of glycosides, alkaloids and flavonoids. The antimicrobial investigation showed that ether extract showed antimicrobial activity against four bacteria and one fungus. From the literature review to was also found that the plant has direct therapeutic effects on piles, dysentery, dyspepsia, diarrhea, vomiting, giddiness, worms, burning of the skin and menorrhoea.

keywords: Antibacterial, Antimutagenic, Menorrhoea, decolorized, saponins, chemotaxonomy, cytotoxic, Isolation.

INTRODUCTION

From the earliest times mankind has used plants in an attempt to cure diseases and relieve physical sufferings. Primitive people in all ages have had some knowledge of medicinal

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plants. Most savage people have believed that disease was due to the presence of evil spirits in the body and could be driven out only by the use of poisonous or disagreeable substances calculated to make the body an unpleasant place in which to remain. As the civilization progressed the early physicians were guided in great part by these observations.(Hill, 1972). The use of plants for curing various human ailments figured in ancient manuscripts such as the Bible, the Rig Veda (4500-1600 BC), the Elliad and the Odyssey and the history Herodotus (Kochhler, 1981). The earliest use of medicinal plants in this subcontinent was evaluated by two Indian authors Charaka and Susruta (Chopra et al, 1956). According to this superstitious doctrine all plants possessed some sign, given with heart- shaped leaves should be used for heart ailments, the sap of blood root (Sanguinaria Canaderisis) as a blood tonic, the liver leaf with its three lobed leaves for liver troubles, the walnut with numerous invaginations and convulsions for brain diseases and pomegranate seeds for dental diseases and so on (Graves, 1990). According to some sources almost 80% of present-day medicines are directly or indirectly derived from plants (Mayers, 1982).Surprisingly, this large quantity of modern drugs comes from less than 15% of the plants, which are known to have been investigated pharmacologically, out of an estimated 250000 to 500000 species of higher plants growing on earth (Farnsworth, et al 1985).). More than 47% of all drugs, used in Russia, are obtained from botanical sources (Ampofo, 1979). At present, thousands of plant metabolites are being successfully used in the treatment of variety of diseases (Farnsworth, et al 1985). A few striking examples of plant metabolites include taxol from Taxus brevifolia (Kumar, et al, 1994), vincristine and vinblastine from Vinca roseus (Satoskar, 1980). all of which are important anticancer agents being used clinically. In the current popular field of chemotherapy, cepharanthine, isolated from Stephanie cepharantha and Stephania sasaki (Jap. Journ. Exp. Med. 1949, 1:69) is being used as a prophylactic in the management of tuberculosis. Even today 30% of rural population of most developing countries of the 70 oce cepends on herbal medicine for maintaining its health and well being (Ghani, 1987). The consumption of medicinal plants is increasing in many seveloped countries, where 35% of drugs contain active principles from natural origin (Irvine, 1995). In China, 150CO factories are involved in producing herbal drugs, herbal medicines have been developed to a remarkable standard by applying modern scientific technology in many countries, such as, China, India, Bangladesh, Srilanka, Thailand and United Kingdom. In these countries, the dependence on allopathic drugs has been decreased to greater extent (Borin, 1998). One hundred and seventy drugs from different plants, which are or once .official in the USP or NF, were

used by the North American Indians. In 1960, 47% of drugs, prescribed by the physicians in the United States of America, were from natural sources (Binge et al., 1960).In 1967, 25% of the products, which appeared in 1.05 billion prescriptions filled in the United States contained one or more ingredients derived from higher plants (Korolkovans, et al. 1966). In the United States, in 1980, the consumers paid 8 billion dollars for prescription drugs in which the active ingredients are still derived from plants (Sofowora, 1982). 47% of some 300 million new prescriptions written by physicians in America in 1961, contained as one or more active ingredients, a drug of natural origin (Famsworth, 1966). Modern medicine still has much to learn from the collector of herbs said Dr. Halfdan Mahler, Director General of the World Health Organization. Many of the plants, familiar to the .witch doctor, really do have the healing power that tradition attaches to them. The age-old art of the herbalist must be tapped (Mayers, 1982). Indeed, the potential of obtaining new drugs from plant sources is so great that thousands of substances of plant origin are being studied for activity against such formidable foes as heart diseases, cancer, and aids (Kirtikar, et al. 1984). In this way, modern medicine will continue to be enriched by the introduction of newer and more potent drugs from plant sources. Therefore, in the present work a humble attempt was made to detect, by using various standard qualitative chemical tests, the presence of reported compounds and to look for possible presence of other chemical constituents in the root and stem bark of the *Bacopa monnieri* (L.) Penn. The Phytochemical screening of the extracts showed the presence of glycosides, alkaloids and flavonoids (Grover et, al 1980). The antimicrobial investigation showed that ether extract showed antimicrobial activity against four bacteria and one fungus. From the literature review to was also found that the plant has direct therapeutic effects on piles, dysentery, dyspepsia, diarrhea, vomiting, giddiness, worms, burning of the skin and menorrhea (Champion,R.H.et,al 1982, the Text book of Darmatology,5th edition,vol-3,p963). The literature survey reveals that the plant has antibacterial and antimutagenic activities. Thus an attempt was made to reinvestigate aforementioned antimicrobial as well as the cytotoxic properties for the bark constituents. The following aspects of *Bacopa monnieri* (L.) Penn., (root bark and stem bark) have been intended to be accomplished in this research project.

MATERIALS AND METHOD

Collection and Preparation of the plant material: The barks (root arid stem) of the plant were collected from campus of Jahangirnagar University during the second week of

December, 2009 by taking great care. Then the barks were separated manually from the undesirable materials. The collected and cleaned barks were cut in to small pieces and then dried in the sun and finally dried in a hot air oven at 50-60°C for 48 hours. After complete drying, the entire portion was reduced to coarse powder with the help of a mechanical grinder. Before grinding, the machine was cleaned to avoid contamination. The dried grinded powder was weighed by using a suitable rough balance. Then the powder was stored in a suitable container for extraction purpose. The cutting, drying, grinding and storage processes were performed for both the root and stem barks.

Extraction of the plant material: The Soxhlet extractor was used for extraction process. The one hundred gram of the dried powder was taken in a porous bag and placed in the Soxhlet chamber. Before placing, the extractor was washed properly and then dried. The powder was soaked with a small portion of the 500 ml ether solvent for some times. Then the rest of the extracting ether was taken in a flask. Upon heating electrically, the vapors were rise trough side arm and condensed in the condenser. The condensed extract was dripped in to the .porous bag containing the plant powder, extracting it by contact. When the level of ether in the chamber was rise to the top of the siphon tube, the liquid contents of the chamber automatically filtered through the powdered containing bag and siphoned into flask. Thus 2000 ml of ether was used successively for four times i.e. 500 ml in each time. Similarly other two solvents ethyl acetate and alcohol were used respectively. The process was performed for the powders of two barks (root bark and stem bark). So three extracts were obtained for each case, named- ether extract, ethyl acetate extract and alcohol extract.

Decolonization and purification: All the extracts were made free from pigments (decolorized) and other impurities by filtration method. This was performed by passing the extracts vyc-J3 activated charcoal on a filter paper and collected in a beaker. The charcoal, used, was made activated by heating in a hot air at 110°C for one hour before filtration. The filter paper was wetted with solvent involved in filtration process. The decolorized and purified extracts were then completely dried by sanction with the help of water bath at a temperature of 50-60° C. Then the extracts were preserved in refrigerator by covering the mouth with paper. Dried decolorized pure extracts were weighed separately with the help of electronic and digital balance and their yield was determined by using the following formula…..

$$\text{Yield} = \frac{\text{Wt. of particular extrac}}{\text{Total amount of coarse powder}} \times 100$$

Phytochemical Tests: Two drops of Molishch reagent were added to about 5 gm of the extract in 5 ml aqueous solution in a test tube 1ml of concentrated H_2SO4 was allowed to flow down the side of the inclined test tube so that the acid formed a layer beneath the aqueous solution without mixing with it. A red ring was formed various phytochemical tests which were performed under heading of phytochemical screening are mentioned below:

i) Molishch's reagent (10% napthol in alcohol)- For carbohydrate test.

ii) Fehling's solution- For glucoside test,

iii) Mayer's reagent (Potassium mercuric iodide solution)- For alkaloid test,

iv) Cone. Hydrochloric acid – For flavonoid test

v) Cone. Sulphuric acid- For steroid test

vi) $FeCl_3$ (5%) – For tannin test.

vii) Solvents- alcohol, chloroform and distilled water.

Small amounts of dried, decolorized extracts were appropriately treated of prepare sample solution and then subjected to various phytochemical test (Grover et, al, 1980)

i) Mollsch's test for carbohydrates: Two drops of Mollsch's reagent were added to about 5 mg of the extract in 5 aqueous solution in a test tube 1 ml of concentrated H_2SO4 was allowed to flow the side of the inclined test tube so that the acid formed a layer beneath solution aqueous section without mixing with it. A red ring was formed at the common surface of the two liquids which indicated the presence of carbohydrate.

ii) Test for glycosides: A small amount of each extract (root and stem sample) was dissolved in water and alcohol then boiled with Fehlling's solution. A brick-red precipitation was noted produced' in this experiment which showed the presence of glycosides in the extract.

iii) Borntrager's test for anthraquinone: 1 ml of each sample (root and stem) was shaken with 5 ml of chloroform in test tube for at least 5 minutes then again shaken with an equal volume of 10% ammonia solution. No bright pink, red or violet color was developed in the aqueous (upper) layer which indicates the absence of free anthraquinones.

iv) Mayer's reagent test for alkaloids*:* Formation of white or cream color precipitate indicated the presence of alkaloids.

v) Test for saponins: About 0.5 ml of each extract was shaken vigorously with water in a test tube. If a frothing was produced and it was stable for 1-2 minutes and persisted on warming, it was taken as preliminary evidence for the presence of saponins but finally it was absence.

vi) Test for flavanoids: A few drops of concentrated hydrochloric acid was added to a small amount of each extract, immediate development of a red color indicated the presence of flavanoids.

vii) Test for steroids: A small amount of each extract was added with 2 ml of chloroform then 1 ml of concentrated sulphuric acid was carefully added from the side of the test tube .In presence of steroids a red color was produced in chloroform layer.

viii)Test for tannis: About 0.5 ml of each extract was stirred with 10 ml of distilled water production of a blue-black ,green or blue-green coloration or precipitation on the addition of $FeCl_3$(5%) regent was taken as evidence for the presence of tannins.

Microbiological Investigation: Study of invitro antimicrobial activity of the alcohol extracts obtained from the extraction of the bark (root and stem) powder of extracts of *Bacopa monnieri* (L.) Penn and rectified spirit**.** The susceptibility of microorganisms to antimicrobial agents could be determined in vitro. The most commonly used method of microbiological assay was the Agar diffusion. In this method, the sample solution was applied on the. piale of antibiotic medium and fungus medium seeded with organisms. After proper incubation the sensitivity of the plant extract was evaluated by measuring the diameter of the inhibition zones produced by the samples. Nutrient agar is a general culture media which may be used as an enrichment medium by incorporating 10% blood or other biological fluid(Sri vastava O.P 1984). Nutrient agar medium was used for the subculture of the test organisms in which proper growth of the organisms were ensured. From this subculture, a very small amount of sample obtained by harvesting was mixed with the antibiotic medium and fungus medium. The formula and the method of preparation of nutrient agar medium were as follows:

Ingredients	gm/liter
Agar	15.00
Beef extract	1.50
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Yeast extract	1.50

To prepare required volume of this medium, amount of each of the constituents were calculated from the composition chart given for I liter. Peptic digest of animal tissue, sodium chloride, beef extract, yeast extract of required calculated amount were taken in a conical flask by weighing them separately. Distilled water was added to it and the content were heated in a water bath to make a clear solution. The pH was then adjusted to 7.4±0.2 using 10% sodium hydroxide solution. Agar was added to the solution in calculated amount and distilled water was added sufficiently to make a final volume. Again the total volume was heated in water bath to obtain a clear solution. The prepared medium was transferred to each of the required number of bottles which were previously washed. The medium was then sterilized by 'autoclaving for 15 minutes at 121°C under a pressure of I5lb/inch^2. Finally the prepared medium was transferred on to a water bath to reduce the temperature 40-50°C.

Antibiotic Assay Medium, Seed Agar: (Hi Media Laboratories' Ltd., India)- This medium was used for bacterial culture to determine the zone of inhibition. The formula and the method of preparation of this medium were as follows:

Ingredients	gm/liter
Peptic digest of animal tissue	6.00
Casein enzymic hydrolysate	4.00
Yeast extract	3.00
Beef extract	1.50
Dextrose	I.OO
Agar	15.00

The required amount of each of the constituents were calculated from the composition chart given for liter and were suspended in required amount of cold distilled water and was boiled for about 15 minutes till complete dissolution. Then the ph of the solution was adjusted to 6.6±0.2 by adding 10% NaOH solution. This medium was then sterilized by autoclaving for 15 minutes at 121° C under a pressure of 15 lb/inch^2. Finally the prepared medium was transferred on to a water bath to reduce the temperature to 40-50°C. For testing the sensitivity of fungus, this medium was used. The composition and the method of preparation of this medium were as follows:

This medium was prepared by dissolving each of the required amount of constituents in distilled water. The solution was boiled for about 15 minutes tilt complete dissolution.

Ingredients	gm/ liter
Yeast extract	5
Nutrient extract Oxoid ^{R} (Code CM_c)	
a. Lab-lemco powder (Oxiod L_{29})	1.0
b. Yeast expract (Oxoid L_{20})	2.0
c. Peptone (Oxoid L_{37})	5.0
d. Agar-3 (Oxoid L_{13})	15
e. Sodium chloride	5

The pH was adjusted to 7.4 by adding 10% sodium hydroxide (NaOH) solution. The medium was then sterilized by autoclaving 'for 15 minutes at 121 °C under a pressure of 15 Ibflnch^2. Finally the prepared medium was transferred on water bath to reduce the temperature to 45-50%.

Test organisms: The bacterial and fungal strains which were used in the sensitivity test are listed in the following table-1. The microorganisms were collected from ICDDRB, Mohakhali, Dhaka and Drug international pharmaceutical Ltd. Dhaka.

Table-1: List of micro-organisms

Serial No.	Microorganisms	Code No.
	Fungus	
1	*Candida albicans*	F-01
	Bacteria	
2	*Salmonella typhi*	B-01
3	*Pseudomonas aeruginos*	B-02
4	*Staphylococcus aureus*	B-03
5.	*Vibrio cholerae*	B-04

Preparation of sample solution: 1 g each of the dried decolorized extracts (ether, ethylacetate and alcohol extracts) were dissolved in 5 ml of distilled water to prepare stock solution. As a solubilizing agent, few drops of 1% Tween-80 was used. So the concentration of the solutions were 200 mg/ml. From these solutions, 200 µl i.e. 2 micropipette (1 micropipette= 0.1 ml) of each sample were, taken and applied into each hole or cup on the agar plate.

To make a comparative study, ampicillin,Gentamicin was taken as a standard as antibiotic and nystatin as antifungal, where the concentration was 200mg/ml.

Inoculation: Specific organisms were inoculated into 30 ml of previously sterilized nutrient agar media with the help of a sterilized inoculating loop. The inoculated medium was mixed thoroughly and immediately transferred to the sterile petridish in an aseptic condition. The petridish was rotated several times, first clockwise then anticlockwise. So that a homogenous distribution of the test organism was prepared. After the completion of preparation of medium, it was stored in an incubator for about 24 hours to allow the proper growth of microbes. After proper incubation the Petridis was harvested by sterile saline TS to prepare bacterial suspension.

Preparation of the cups: The temperature of sterilized antibiotic medium was reduced to 45-50°C and 100 ml of it was transferred in a bottle. A small volume of bacterial suspension (1-2 ml) was added to the bottle containing l00 ml antibiotic medium.. After proper mixing, a suitable volume (25-30 ml) of this medium was added to a sterilized plate to a layer of uniform thickness (2-3mm.). After solidification of the seeded antibiotic medium, small holes or cups were cut in it with the help of a specific borer. Four types of cups were prepared in each plate and they were-Alcohol extract cup ,Alcohol cup, Standard cup (ampiciffin/nystatin cup),Standard cup(Gentamicin cup). The cups were sufficiently far from each other to prevent overlapping of the zones of inhibition. Prepared sample solutions were applied to the corresponding cups or holes with the help of a micropipette. The plates were then allowed to stand to diffuse the sample solution font the antibiotic medium at room temperature for an hour so that the diffusion of the sample solutions was completed. The plates were then incubated at 37°C for overnight.

RESULT AND DISCUSSION

Phytochemical evaluation: The studies of as mentioned before 100 gm of coarsely plant materials were extracted successively with rectified sprite (alcohol) .Yield of the extracted are shown of the flowing tables-2.

Table -2: Yield of the extracted of Bacopa monniera

Part used	Dried materials(g)	Amount yield(ml)	Yield
Whole herb and leaves	200	6	3%

Qualitative Phytochemical test were for all the extracts alchol extracted of the plants Bacupa monniera. The result of various qualitative chemicals tests for the detection and identification of chemical constituents' of Bacupa monniera are summarized by the given table.

Table- 3: The result of various qualitative chemicals tests for the detection and identification of chemical constituents' of Bacupa monniera

Test for	Alcohol extracted
Carbohydrates	+
Glycosides	+
Anthra-quine Glycosides	-
Alkaloids	+
Saponines	-
Flavonoids	+
Steroids	+
Tennins	+

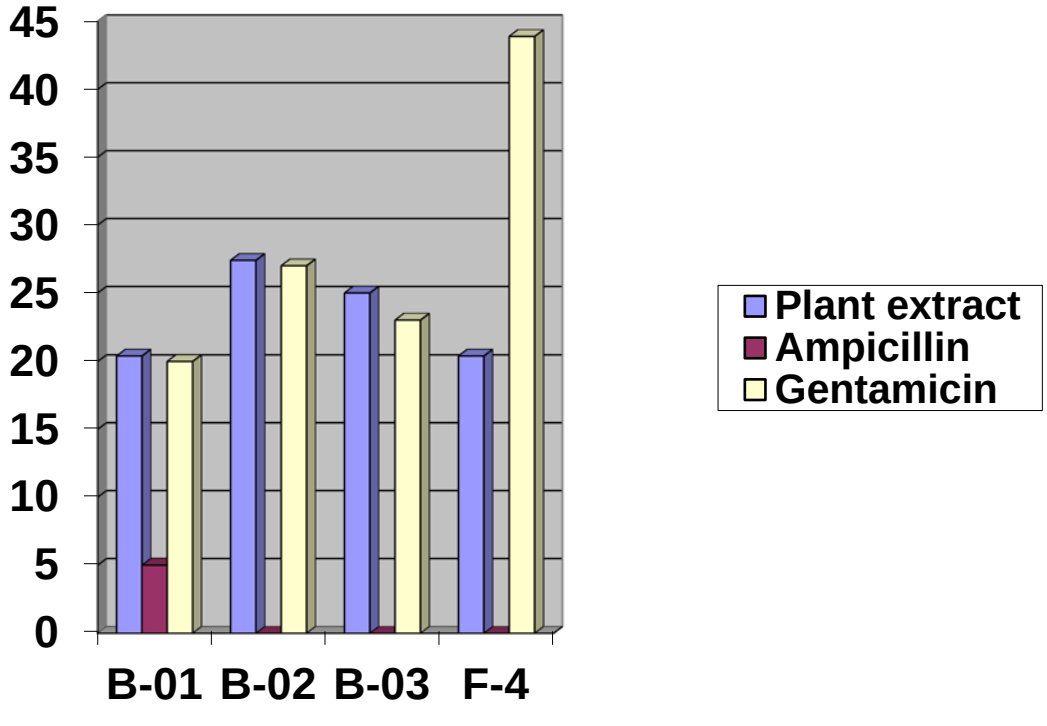
"+" =Positive Reaction, "-"=Negative Reaction

The alcohol of herb part of the plant Bacopa monniera gave positive reaction for carbohydrate , Alkaloids, Steroids, Tennins and negative response for Saponines and Anthra-quineglycosides. As the chemical constituents present in a plant are directly responsible for its therapeutic and other pharmacological properties the constituents of the plants which are reported and detected during the investigation should have some direct relationship with local medicinal plant uses. Approximately all the samples of Bacupa monniera have been found to contain Carbohydrate Steroids Flavonoids Alkaloids tennins. Carbohydrates are used in pharmacy mainly as pharmaceutics necessities sush as suspending and emulsifing agent (Tragacanth,acacia ,agar ,alginantes)thikening agent diluents and distegrant (Starch).The steroids have antipyretic and analgesic effects . Alkaloids being bitter substance exert notable antimicrobial action. So it is quit reasonable that the plant containing alkaloids exert beneficial therapeutic effects aginest infections diseases for which it is used. Tinines are only remarkable for trhere antiseptic property but also for there astringent action. This astringent properties afford them the therapeutic value in arresting hemorrhage by constructing blood vessels and in protecting wounds, inflamation and ulcer from external irritation by the participating surface protein which from impervious coating on them. Thus it is evident tht the constituents (Alkaloids ,tannins) are sufficient to cure infection and tannins

are also responsible to cure inflammatory diseases. As apparent from results and other supportive evidences local use of the plant extracts against various diseases like inflammation ,infection ,ulcers ,fever ,dysentery are not much variance with there chemical constituents.

Microbiological evolution: Antimicrobial Activities of the decolorized extracts of *Bacopa monnieri* (L.) Penn. The in vitro antimicrobial activities of the root extract were tested against 8 pathogenic bacteria and 1 fungus which are responsible for various infection disease .Every plate are 4 holes are made one of plant extract two holes are antibiotic and one are rectified spirit which are explain by the bar graph

Sample code	Microorganism	Inhibition zone
B-01	Salmonella typhi	21
B-2	Staphyloccous aureus	19
B-3	Bacilluscaereus	18
B-4	Pseudomonas aeruginos	00
B-5	Klebsiella species	00
	Fungus	
F-1	Candida albicans	16
s-1	Organism collected from soil	17



Concentration of plant extract =200,Concentration of Gentamicine =200,Concentration of Ampiciline =200. The Sensitivity of these microbes to the plant extract and compression with a standard antibiotic Gentamicin and Ampiciline shown in flowing bar graph and figure

CONCLUSION

The powdered plant was extract with alcohol. Phytochemical screening of the extract showed the presence of alkaloids ,Glycosides and flavanoides. The antimicrobial activities of the decolorized extracts were invested against four pathogenic bacteria and fungus .Alcohol extract showed significant antimicrobial activities.

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