IJPSR (2012), Vol. 3, Issue 09 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 18 May, 2012; received in revised form 27 July, 2012; accepted 27 August, 2012

PHYTOCHEMICAL SCREENING AND *IN VITRO* ANTIMICROBIAL INVESTIGATION OF THE METHANOLIC EXTRACT OF *CENTELLA ASIATICA* LEAVES

Rishikesh*[1], Md. Mofizur Rahman [1], S.M. Siddiqul Islam [2] and Md. Moshfiqur Rahman [2]

Department of Pharmacy, Bangladesh University [1], Dhaka-1209, Bangladesh
Department of Pharmacy, Manarat International university[2], Bangladesh

Keywords:
In vitro Antimicrobial,
Centella asiatica,
Methanolic Extract,
Phytochemical Screening

Correspondence to Author:

Rishikesh

B. Pharm, MS. Pharm. Tech., Department of Pharmacy, Bangladesh University, Dhaka-1207, Bangladesh

E-mail: rishibd@gmail.com

ABSTRACT

The methanol extract of *Centella asiatica* (Family:Umbelliferae) was studied for its phytochemical and pharmacological Activities. This study of the extracts indicates the presence of Reducing Sugar Tannins, Alkaloids, Sapponins and.The pharmacological study include Antimicrobial activities.The extract of the plant showed activity against a wide a variety of microorganism's .All the activities were compared by measuring the zone of inhibition with the standard antibiotic. Based on the findings of Antimicrobial activity, we can say that the plant may contain novel compounds that possess potent anti-mutagenic activity and anti-oxidative activity. The obtained results support for the uses of this plant as traditional medicine. Infectious diseases are the second leading cause of death worldwide. Treatment of infections continues to be problematic in modern time because of the severe side effects of some drugs and the growing resistance to antimicrobial agents. The activities observed could be attributed to the presence of some of the phytochemicals detected which have been associated with antibacterial activity.

INTRODUCTION: Bacteria and fungi are responsible for many infectious diseases. The increasing clinical implications of drug resistant fungal and bacterial pathogens have lent additional urgency to antimicrobial drug research. Medicinal plants are major sources of obtaining antimicrobial agents [1]. Plants are used medicinally worldwide as sources of many potent drugs [2].Traditional medical practitioners use variety of herbal preparations to treat different kinds of diseases including microbial infections [3].

Plants are the natural reservoir of many antimicrobial agents. In recent times, traditional medicine as an alternative form of health care and to overcome microbial resistance has led the researchers to investigate the antimicrobial activity of medicinal plants [4]. The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth.

This ability may be estimated by any of the following three methods;

QUICK RESPONSE CODE

IJPSR: ICV- 4.57

Website: www.ijpsr.com

1. Disc diffusion method
2. Serial dilution method
3. Bioautographic method

But there is no standardized method for expressing the results of antimicrobial screening [5]. Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods [6], inoculum volume, culture medium composition [7], p^H [8], and incubation temperature [9] can influence the results.

Among the above mentioned techniques the disc diffusion [7] is a widely accepted in vitro investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method [10].

The use of medicinal plants as a source for relief from illness can be traced back over five millennia to written documents of the early civilization in China, India and the Near east, but it is doubtless an art as old as mankind [11]. Antibiotics are one of our most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction. However, over the past few decades these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not only because many of them produce toxic reactions but also due to emergence of drug-resistant bacteria. It is essential to investigate newer drugs with lesser resistance [12].

MATERIALS AND METHODS

Method: The Phytochemical investigation of plant can be divided roughly into following major steps:

a) Collection.

b) Preparation of plant extract.

c) Extraction.

d) Isolation of compounds.

e) Structure elucidation and characterization o compounds.

Collection and identification of the plant sample: The plant centella asiatica (Family: Umbelliferae) was collected from Dhaka and was taxonomically identified with the help of National Herbarium of Bangladesh, Dhaka. The leave and the steam were cut into small pieces and the sun dried for seven dayes.The leaves and stems were ground into coarse powder with the help of an attrition type of a grinder.

Extraction of leaves: About 250 gm of powdered leaves was taken in a clean flat-bottomed glass container and percolated with 3 liters of Methanol. The container with its content was sealed and kept for 7 days with occasional shaking and stirring .the mixture was the filtered successively through a piece of clean white cotton .The filtrate thus obtained are kept in a open air for the evaporation of the methanol. After 10 to 15 days all the methanol are evaporated and I got the extract of methanol

Extraction at a glance:

Plant collection
↓
Plant identification
↓
Bulk collection
↓
Drying
↓
Maceration in methanol
↓
Filtration by cotton plug
↓
Filtration by Whatman filter paper
↓
Evaporation
↓
Crude methanol extract

Identification of chemical group [13, 14]: Testing of difference chemical group present in extract represent the preliminary photochemical studies. The chemical group test, which are performed as follows in each test 10%(w/v) solution of extract in methanol was taken.

Reagent used for the different chemical group test [13, 14, 15, 16]: The reagents were used for the different chemical group test.

1. **Mayer's reagent:** 1.36 gm mercuric iodide in 60 ml of water was mixed with a solution contains 5 gm of potassium iodide in 20 ml ml water.

2. **Dragendroff's Reagent:** 1.7 gm basic bismuth nitrate and 20 gm tartaric acid ware dissolved in 80 ml water. This solution was mixed with a solution contain 16 gm potassium iodide and 40ml water.

3. **Fehling solution A:** 34.464 gm copper sulphate was dissolved in a mixture of 0.50 ml of sulfuric acid and sufficient water to produce 500 ml.

4. **Fehling solution B:** 176 gm of sodium potassium tartarate and 77 gm of sodium hydroxide were dissolved in sufficient water to produce 500 ml . Equal volume of above solution were mixed at the time of use.

5. **Benedicts Reagent:** 1.73 gm of sulphate 1.73 gm sodium citrate and 10 gm anhydrous sodium carbonate were dissolved in water and the volume was made up to 100 ml with water.

6. **Molish Reagent:** 2.5 gm of pure α – naphthol was dissolved in 25 ml of methanol

7. **Liebermann- Burchard Reagent:** 5 ml of acetic anhydrous was carefully mixed under cooling with 5 ml concentrated sulfuric acid. This mixture was added cautiously to 50 ml absolute methanol with cooling.

Test for reducing sugar:

1. **Benedict's Test:** 0.1 ml aqueous extract of the plant material was taken in test tube . 1ml of benedict's solution was added to the test tube , boiled for 5 minutes and allowed to cool spontaneously red color precipitate of cuprous oxide was formed in the presence of a reducing sugar.

2. **Fehling's Test (Standard test):** 2 ml of an aqueous extract of the plant material was added 1 ml of a mixture of equals volume of Fehling solution A and B .Boiled for minutes. A red or brick red color precipitate was former that indicate that reducing sugar is present.

3. **Alpha Naphthol solution test:** 0.5 ml solution of extract faded with 2 drops of 5%Alpha- Naphthol solution (Freshly prepared) and added0. 1 ml of sulfuric acid on the sides of the test tube .Violet colored ring was formed at junction of two liquid in the **presence** of reducing sugar.

Test for tannins:

1. **Ferric chloride Test:** 0. 5 ml of solution of extract was taken in a test tube. Then 0.1 ml of 5% Ferric chloride solutions was added. Greenish black precipitate was formed and indicated the presence of tannins.

2. **Potassium dichromate test:** 0.5 ml solution of extract was taken in a test tube .Then0.1 ml of 10% Potassium dichromate solution was added. A yellow precipitate was formed in the presence of tannins.

Test for Flavonoids: Added a few drops of concentrated hydrochloric acid to a small amount of an alcoholic extract of the plant material. Immediate development of a red colors indicates presence of Flavonoids

Test for Saponnins: 1 ml solution of extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. One – centimeter layer of foam indicates the presence of saponnins

Test for Gums: 5 ml of extract was taken in the molish reagent and sulphuric acid were added. Red violet ring produced at the junction of two liquid indica the presence of gums and carbohydrate.

Test for steroids:

1. **Liebermann- Burchard test:** 1ml solution of chloroform extract was taken and then added 2 ml Liebermann-Burchard reagent. Reddish purple color indicates the presence of steroid.

2. **Sulphuric acid test**: 1 ml of solution chloroform extract was taken and then added0.2 ml Sulphuric acid . Red color indicates the presence of steroids

Tests for Alkaloids:

1. **Mayer's Test:** 2 ml of solution of extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Myer's reagent were added. Yellow color precipitate was formed and that was indicated as the presence of alkaloids.

2. **Dragendroff's test:** 2 ml of solution of extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Dragendroff's reagent was added. Orange brown precipitate was formed and that was indicated as the presence of alkaloids. The alkaloidal fraction of the stem has also been reported to be active against all micro-organisms (Abo and Ogunleye, 1999).

3. **Wagner's test:** 2 ml of solution of extract and 0.2 ml of dilute hydrochloric acid were taken in a test Tube. Then 1 ml of iodine solution (Wagner's reagent) was added. Reddish brown precipitate was formed and that was indicated as the presence of alkaloids

4. **Hager's test:** 2 ml of solution of extract and 0.2 ml of dilute hydrochloric acid were taken in a test Tube. Then 1 ml of of picric acid solution (Hager's reagent) was added. Yellowish precipitate was formed and that was indicated as the presence of alkaloids.

Antibacterial activity:

Principle of Disc Diffusion Method: In this classical method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Dried and sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (Ciprofloxacin) discs and blank discs are used as positive and negative control. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion of the test materials to the surrounding media [18]. The plates are then inverted and incubated at 37°C for 24 hours for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimeter [7, 18].

In the present study the crude extracts, fractions as well as some pure compounds were tested for antimicrobial activity by disc diffusion method. The experiment is carried out more than once and the mean of the readings is required (Bayer *et al.,* 1966).

Experimental:

Apparatus and Reagents: Filter paper discs, Petri dishes, inoculating loop, Sterile cotton, sterile forceps, Spirit burner, Micropipette, Screw cap test tubes, Nose mask and Hand gloves, Ethanol, Incubator, Chloroform, Autoclave, Refrigerator, Nutrient Agar Medium, Laminar air flow hood.

Test Organisms: The bacterial and fungal strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Both gram positive and gram-negative organisms were taken for the test and they are listed in the **Table 1**.

TABLE 1: LIST OF TEST BACTERIA AND FUNGI:

Gram positive Bacteria	Gram negative Bacteria	Fungi
Bacillius sereus	*Escherichia coli*	*Candida albicans*
Bacillius megaterium	*Pseudomonas aeruginosa*	*Aspergillus niger*
Bacillius subtilis	*Salmonella paratyphi*	*Sacharomyces cerevacae*
Staphylococcus aureus	*Salmonella typhi*	
Sarcina lutea	*Shigella boydii*	
	Shigella dysenteriae	
	Vibrio mimicus	
	Vibrio parahemolyticus	

Culture medium and their composition: The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms. Prepared Muller-Hunton medium was supplied from IPH (Institute of Public Health) its composition/100 ml is shown here;

Muller – Hunton medium:

Ingredients	Amounts
Beef infusion	30 gm
Casamino acid	1.75 gm
Starch	0.15 gm
Bacto agar	1.70 gm
Distilled water q.s.	100 ml
pH	7.3 ±0.2 at 250 C

Nutrient agar medium (DIFCO) is the most frequently used and also used in the present study for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures.

Preparation of medium: To prepare required volume of this medium, calculated amount of Muller-Hinton agar was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The pH (at 25^{o}C) was adjusted at 7.2-7.6 using NaOH or HCl. 10 ml and 5 ml of the medium. The conical flasks were then capped and sterilized by autoclaving at 15-lbs. pressure at 121^{o}C for 20 minute. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

Sterilization procedures: In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petri dishes and other glassware were sterilized by autoclaving at a temperature of 121^{o}C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

Preparation of subculture: In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37^{o}C for their optimum growth. These fresh cultures were used for the sensitivity test.

Preparation of the test plates: The test organisms were transferred from the subculture to the test tubes containing about 10 ml of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial suspension was immediately transferred to the sterilized Petri dishes. The Petri dishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media [19, 20].

Preparation of discs: Three types of discs were used for antimicrobial screening.

Standard discs: These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, Ciprofloxacin (30μg/disc) standard disc was used as the reference.

Blank discs: These were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

Preparation of sample discs with test samples: Measured amount of each test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank Petri dish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

Preparation of sample discs with test samples of *Centella asiatica*: Measured amount of each test sample (specified in table 7.2) was dissolved in specific volume of solvent (ethanol) to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank Petri dish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

Preparation of Sample Discs:

Plant	Sample	Dose (μg/disc)	Required amount for 6 disc (mg)
Centella asiatica	Hexane soluble fraction of Methanolic extract	400	10
	CCl_4 soluble fraction of Methanolic extract	400	10
	Methanolic extract of whole plant	400	10
	Aqueous fraction of Methanolic extract	400	10

Application of the Test Samples: Standard Ciprofloxacin (30 μg/disc) discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Blank discs were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

Diffusion and Incubation: The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4°C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37°C for 24 hours.

Determination of Antimicrobial Activity by measuring the Zone of Inhibition: The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale [21, 22].

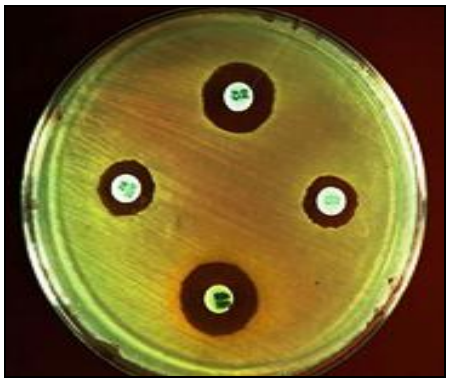

FIG. 1: CLEAR ZONE OF INHIBITION

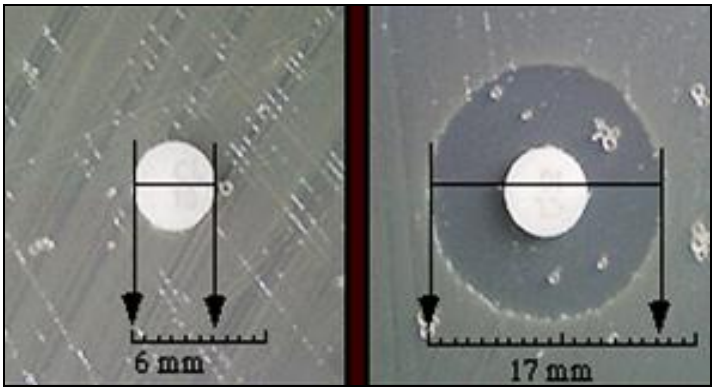

FIG.: DETERMINATION OF CLEAR ZONE OF INHIBITION

Results and Discussion of *in vitro* Antimicrobial screening of *Centella asiatica*: The hexane, carbon tetrachloride, dichloromethane soluble fraction of the Methanolic extract and crude Methanolic extract exhibited antimicrobial activity against most of the test organisms. The zones of inhibition produced by carbon tetrachloride, *n*-hexane and deichloromthane soluble fraction of the Methanolic extract showed average zones of inhibition (10 mm), (9 mm) and (9 mm) respectively at a concentration of 400 μg/disc.

The crude Methanolic extract of the bark showed antimicrobial activity against the test organisms, average zone of inhibition (10.5mm)

From the table given below, we can see the zones of inhibition produced by carbon tetrachloride, ethanol extract, n-hexane soluble fraction of the Methanolic extract showed average zones of inhibition 7.62mm, 7.56mm, 0 mm respectively at a concentration of 400 μg/disc. And for standard the average zones of inhibition was 41mm. So in comparison with standard (Ciprofloxacin), the Methnolic extract, n-hexane, carbon tetrachloride soluble fraction of the Methanolic extract exhibited mild antimicrobial activity against most of the test organisms [23, 24].

TABLE 2: ANTIMICROBIAL ACTIVITY OF EXTRACTS AND DIFFERENT FRACTIONS OF *CENTELLA ASIATICA.*

Test microorganisms	Diameter of zone of inhibition (mm)			
	CCl_4	Methanolic Extracts	n-hexane	Ciprofloxacin
Gram positive bacteria				
Bacillius sereus	08	08	--	40
Bacillius megaterium	08	08	--	41
Bacillius subtilis	09	09	--	40
Staphylococcus aureus	08	08	--	40
Sarcina lutea	08	08	--	40
Gram negative bacteria				
Escherichia coli	08	08	--	42
Pseudomonas aureus	08	08	--	40
Salmonella paratyphi	09	08	--	40
Salmonella typhi	08	08	--	40
Shigella boydii	08	08	--	40
Shigella dysenteriae	08	08	--	40
Vibrio mimicus	08	08	--	40
Vibrio parahemolyticus	08	08	--	40
Fungi				
Candida albicans	08	08	--	41
Aspergillus niger	--	--	--	41
Sacharomyces cerevacae	08	08	--	41

HFL= n-Hexane soluble fraction of extracts, CCl_4 =Carbon tetrachloride soluble fraction of extracts. 8mg dissolved in (200μl ethanol) per disc 10μl concentration 400 μg

DISCUSSION: Plants are important source of potentially useful structures for the development of new antimicrobial agents. The first step towards this goal is the *in vitro* antibacterial activity assay. Many reports are available on the antiviral, antibacterial, antifungal and anti-inflammatory properties of plants. Some of these observations have helped in identifying the active principle responsible for such activities and in the developing drugs for the therapeutic use in human beings [11].

However, not many reports are available on the tannins have been traditionally used for protection of Inflamed surfaces of the mouth and treatment of catarrha, wounds, haemorrhoids, and diarrhea, and as antidote in heavy metal poisoning. Flavonoids are naturally occurring phenols which possess numerous biological activities including anti-inflammatory, antiallergic, anti-thrombotic and vasoprotective effects. Cyanogenetic glycosides are reported to possess antimicrobial activity. The observed antimicrobial activity against the tested organisms could be due to the presence of tannins and cyanogenetic glycosides in the extract as these have previously been reported to possess antimicrobial activities.

These could explain the rationale for the use of the plant in the treatment of the various conditions in traditional medical practice [20]. The usage of herbal plants as traditional health remedies is the most popular for 80% of the world population in Asia, Latin America and Africa and is reported to have minimal side effect [25]. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies including medicinal herbs [26].

CONCLUSION: After measuring the diameter of the zones of inhibition in millimeter with a transparent scale, the ethnol extract, n-hexane, carbon tetrachloride soluble fraction of the Methanolic extract exhibited high antimicrobial activity against gram positive and gram negative bacteria.

We therefore, suggest further, the purification and characterization of the phytochemicals that would be obtained with a view to obtaining useful chemotherapeutic agent.

ACKNOWLEDGEMENTS: We are grateful to our honorable chairman Sri. Dr. Md. Anwarul Islam. The authors are also thankful to Bangladesh University, Manarat International university of Dhaka for their supports and cooperations

REFERENCES:

1. A Sarkar, KA Kumar, NK Dutta, P Chakraborty, SG Dastidar: Evaluation of In vitro and In vivo antibacterial activity of dobutamine hydrochloride. Indian Journal of Medical Microbiology; 2003;21 (3):172-178
2. Abo, K.A., Ogunleye, V.O. and Ashidi, J.S. (1999). Antimicrobial potential of *Spondiasmombin, Croton zambesicus* and *Zigotritonia crocea. Phytother. Res*. 13 (6) 494-497.
3. Austin. D.J., Kristinsson. K.G., Anderson.R.M. 1999. The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. Proc Natl Acad Sci USA., 96:1152-6.
4. Ayafor,J.F.,Sondengam,B.L.,Ngadjui,B.T.(1980).Veprisine and N-Methylpreskimmianine: novel 2-quinolones from Vepris louisii Tetrahedron Letters 21, 3293-3294.
5. Baver. A.W., Kirby. M.D.K., Sherris. J.C, Turck. M. 1966. Antibiotic susceptibility testing by standard single disc diffusion method. Am. J. Clinical pathol., 493-496.
6. Bauer, A.W., *et al*. (1966): Am. J. Clin. Pathol. 45:493-496.
7. Barry, Lea & Febiger, 1976 - Medical - 236 pages, Manual of clinical microbiology ... Anti-infective agents.
8. B. Mahesh and S. Satish; Antimicrobial Activity of Some Important Medicinal Plant against Plant and Human Pathogens: World J. Agric. Sci. 2008; 4 (S): 839-843.
9. Chopra RN, Chopra 1C, Hunda KI and Kapur LD, Chopra's Indigenous Drugs of India, Academic publishers, New Delhi, India, (1982), 1-15.
10. Chopra, R.N., Nayar, S.I. and Chopra, I.E., Glossary of Medicinal Plants, Council of Scientific and Industrial Research, (1956), 57.
11. D. S. Ogunleye and S. F. Ibitoye; Studies of antimicrobial activity and chemical Constituents of Ximenia Americana; Trop J Pharm Res. December 2003; 2 (2): 239-241
12. Dev.S, Phytochemical and Pharmacological Studies on Aibizzia lebbeck (family: Mimosaceae), B.Pharm project thesis submitted to discipline of Khulna University, (2002).
13. Ghani, A, Medicinal Plants of Bangladesh, published by Asiatic Society, 1st edition, (1998), 13.
14. Ghani, A., Medicinal Plants of Bangladesh: Chemical constituents & uses. Asiatic Society of Bangladesh.2nd edition, (2003), 1-16,381
15. Ghani, A., Traditional Medicine, Jahangirnagar University, Savar, Dhaka. (1990), 26-27, 39-40.
16. Ghani, A., Practical Phytochemistry, Jahangirnagar University, Savar, Dhaka, (1997), 84-89.
17. Iwu MW, Duncan AR, Okunji CO (1999). New Antimicrobials of Plant origin In: J. Janick (ed.), Perspectives on New Crops and New Uses. ASHS Press, Alexandria, VA. 457-462
18. Kudi, A.C., J.U. Umoh, L.O. Eduvic, J. Getu, (1999). Screening of some Nigerian Medicinal plants for Antibacterial Activity. *J. Ethanopharm*. 67:225-228.
19. Leven M, Vanden-Berghe DA, Morten I, Vilientrick A, Lomweos EC (1979). Screening of higher plants for biological activity. Planta. Medica. 36: 311-312.
20. Lorian V (1991). Antibiotics in Laboratory Medicine: Laboratory methods used to assess the activity of antimicrobial combinations, 3rd ed., Williams and Wilkins, Baltimore, 434-444.
21. May T. Nadir, Natiq A.R. Hatam, Nada Abdul-Khaliq, and Nahia J. Yousif ,The Constituents of Achillea conferta: Phytochemical and Antimicrobial Studies, pharmaceutical biology, 1991, Vol. 29, No. 2 : 89-93
22. M. A. Ekpo* and P. C. Etim: 'Antimicrobial activity of ethanolic and aqueous extracts of Sida acuta on microorganisms from skin infections.' Journal of Medicinal Plants Research. September, 2009; 3(9): 621-624, Available Online at http://www.academicjournals.org/JMPR.
23. Roland R. 1982. Antibiotics: An Introduction. F. Hoffman La Roche and Co. Basle, Switzerland. 70-71.
24. Sofowora A (1986). Medicinal plant and traditional medicine in Africa II, John Wiley Chiechester. 178.
25. Sofowora A (1993). Medicinal and Traditional medicine in Africa. Second edition, published by Spectrum Books Limited, Ibadan,Nigeria. 130.

How to cite this article:
Rishikesh, Rahman MM, Islam SMS and Rahman MM: Phytochemical Screening and *In vitro* Antimicrobial Investigation of the Methanolic Extract of *Centella asiatica* Leaves**.** *Int J Pharm Sci Res*, 2012; Vol. 3(9): 3323-3330.