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Research Article

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Phytochemical and antibacterial efficacy of *Hevea brasiliensis*

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ABSTRACT

It is now believed that nature has given the cure of every disease in one way or another. Plants have been known to relieve various diseases in Ayurveda and Siddha. Therefore, the researchers today are emphasizing on evaluation and characterization of various plants and plant constituents against a number of diseases based on their traditional claims of the plants given in traditional system of Indian medicine. Extraction of the bioactive plant constituents has always been a challenging task for the researchers. In this present study, an attempt has been made to give an overview of certain extractants and extraction processes of Hevea brasiliensis and is of interest to analyse the various phytochemical constituents of butyl alcohol, Acetone and Chloroformic extracts of Hevea brasiliensis. Our results indicate that the medicinal plant Hevea brasiliensis posses secondary metabolites such as flavanoids, alkaloids, tannins, cardiac glycosides and steroids etc., Therefore, it is of interest to investigate the phytochemical and the antibacterial effects of Hevea brasiliensis against E.coli, Pseudomonas aeruginosa and Klebsila pneumoniae

Key words: *Hevea brasiliensis*, Ethanol, Chloroform, Phytomedicine, Alkaloids, Flavonoids, *E.coli, Pseudomonas Aeruginosa and Klebsila pneumoniae*

INTRODUCTION

Plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. Nowadays, the use of phytochemicals for pharmaceutical purpose has gradually increased in many countries. According to World Health Organization (WHO) medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants.[1]

The use of crude extracts of plants parts and phytochemicals, of known antimicrobial properties, can be of great significance in the therapeutic treatments. In recent years, a number of studies have been conducted in various countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to the secondary metabolites synthesized by the plants. These products are known by their active substances like, phenolic compounds which are part of the essential oils, as well as in tanning. The screening of plant products for antimicrobial activity have shown that the higher plants represent a potential source of novel antibiotic prototypes There has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This has forced scientist to search for new antimicrobial substances from various sources like the medicinal plants [2].

Plant produces a wide variety of secondary metabolites which are used either directly as precursors or as lead compounds in the pharmaceutical industry. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant microbial pathogens. However, very little information is available on such activity of medicinal plants and out of the 4,00,000 plant species on earth, only a small number has been systematically investigated for their antimicrobial activities. [3] *Hevea brasiliensis*, the Pará rubber tree or sharinga tree, or, most commonly, the rubber tree, is a tree belonging to the family Euphorbiaceae. It is the most economically important member of the genus *Hevea*. It is of major economic importance because the milkylatex extracted from the tree is the primary source of natural rubber.

Hevea brasiliensis

EXPERIMENTAL SECTION

Plant Materials

The medicinal plant used for the experiment were leaves of *Hevea brasiliensis*. collected from local ayurvedic shop, and the plant material were identified and authenticated by botanist Chennai, Tamil nadu. India.

Extraction from plants

500 grams of leaf of *Hevea brasiliensis* .plant was packed in separate round bottom flask for sample extraction. The extraction was conducted by 1000 ml of the solvent mixture for a period of 72 hours. At the end of the extraction the

respective solvents were concentrated under reduced pressure and the crude extracts were stored in refrigerator, for further use.

Phytochemicals analysis
The extracts prepared were analyzed for the presence of alkaloids, saponins, tannins, steroids, flavonoids, anthra quinones, cardiac glycosides and reducing sugars based on the protocols available in the literature. [4]

Test for alkaloids
The extract of the crude dry powder of each solvent was evaporated to dryness in boiling water bath. The residues were dissolved in 2 N Hydrochloric acids. The mixture was filtered and the filtrate was divided into three equal portions. One portion was treated with a few drops of Mayer's reagent, one portion was treated with equal amount of Dragondroff's reagent and the third portion was treated with equal amount of Wagner's reagent respectively. The appearance of creamish precipitate, the orange precipitate and brown precipitate indicated the presence of respective alkaloids. [5]

Test for saponins
About 2 ml of plant leaf extract was vigorously shaken with water in a test tube and then heated to boil. Frothing was observed which was taken as a preliminary evidence for the presence of the saponins

Test for tannins
About 2 ml of plant leaf extract was added was in 10 ml of water in a test tube and filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or blue-black coloration. [6]

Test for steroids
2 ml of acetic anhydride was added to 2 ml of plant extract of each sample along with 2 ml sulphuric acid. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

Test for flavonoids
2 ml of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution few drops of conc. Hydrochloric acid was added and the red colour was observed for flavonoids and orange colour for flavones. [7]

Test for anthraquinones
About 2 ml of extract was taken in a dry test tube and 5 ml of chloroform was added and shaken for 5 min. The extract was filtered and the filtrate shaken with equal volume of 10% of ammonia solution. A pink violet or red colour in the ammonical layer indicates the presence of anthraquinones**.** [8]

Test for cardiac glycosides
2 ml of extract was dissolved in 1 ml of glacial acetic acid containing 1 drop of ferric chloride solution. This was then under layered with 1ml of concentrated sulphuric acid. A brown ring obtained at the interface indicated the presence of a deoxysugar characteristic of cardioids. [9]

Test for Proteins
To 2ml of extract and 1 ml of protein solution 1ml of 40% NaOH solution and 1 to 2 drops of 1% $CuSO_4$ solution was added. A violet colour indicated the presence of peptide linkage of the molecule**.** [9,10]

Test for Amino Acids
To 2 ml of sample was added to 2 ml of Ninhydrin reagent and kept in water bath for 20 minutes. Appearance of purple colour indicated the presence of amino acids in the sample. [9]

Test for Tri-Terpenoids
5ml of each extract was added to 2ml of chloroform and 3ml of con. H_2SO_4 to form a monolayer of reddish brown coloration of the interface was showed to form positive result for the tri-terpenoids. [9]

Test for Reducing Sugar
To 2 ml of extract 2 drops of Molisch's reagent was added and shaken well. 2ml of conc. H_2SO_4 was added on the sides of the test tube. A reddish violet ring appeared at the junction of two layers immediately indicated the presence of carbohydrates**.** [10]

Antibiotics
Broad spectrum antibiotics, Penicillin and Ampicillin were used as control drugs.

Bacterial Strains
The strains of microorganisms (*E.coli, Klebsila pneumoniae and Pseudomonas aeruginosa* were used.

Determination of Antimicrobial Activity
Antimicrobial activity was measured using the standard method of diffusion disc plates on agar and the MIC was calculated using dilution method (Kirby- Bauer method).

Dilution Methods
Dilution susceptibility testing methods were used to determine the minimal concentration of antimicrobial to inhibit or kill the microorganisms. This was achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials are generally tested in log2 serial dilutions (two fold).

Broth Dilution Method
The Broth Dilution Method is a simple procedure for testing a small number of isolates, even single isolates.

Preparation of microorganisms for experiment
The pure cultures of organisms (*E.coli, Pseudomonas aeruginosa and Klebsila pneumoniae)* were sub-cultured in nutrient broth.They were inoculated, separately, into nutrient broth and kept at 37°C for 24 hours. Then, they were kept at 4°C until use.

Growth Method
At least three to five well-isolated colonies, of the same morphological type, were selected from an agar plate culture of a particular microorganism. The top of each colony was touched with a loop, and the growth was transferred into a tube, containing 4 to 5 ml of Nutrient broth medium. The broth culture was incubated at 35°C for 8 hours. After the incubation period broth culture became turbid.

Disc Diffusion Method:
Mueller-Hinton Agar Medium
Mueller-Hinton Agar is considered to be the best for routine susceptibility testing of non- fastidious bacteria for the following reasons; It shows acceptable batch-to-batch reproducibility for susceptibility testing. Medium is transparent, so that the inhibition zone can be visualized clearly. It gives satisfactory growth of most non fastidious pathogens. A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

Preparation of Mueller-Hinton Agar
Mueller-Hinton Agar was prepared from a commercially available dehydrated base according to the manufacturer's instructions. Immediately after autoclaving, it was allowed to cool in a 45 to 50°C water bath. The freshly prepared and cooled medium was poured into glass or plastic, flat-bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30 ml for plates with a diameter of 100 mm. The agar medium was allowed to cool to room temperature and unless the plate is used the same day, stored in a refrigerator. Plates were used within seven days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimize drying of the agar. A representative sample of each batch of plates was examined for sterility by incubating at 30 to 35°C for 24 hrs or longer.

Preparation of antibiotic stock solutions
Powders of the two antibiotics (Penicillin and Ampicillin) were brought from authorized medical shop. They were accurately weighted and dissolved in sterile distilled water in appropriate dilutions to yield the required concentrations. The stocks were kept in aliquots of 5 ml volumes and frozen at -20°C

Preparation of plant extract solutions for the experiment
The dried plant extracts were weighed and dissolved in sterile distilled water to prepare appropriate dilution to get required concentrations (1.0mg/ ml, 1.5mg/ ml, 2.0 mg/ ml, and 2.5 mg/ ml). They are kept under refrigeration.

Preparation of dried filter paper discs
Whattman filter paper (No.1) was used to prepare discs approximately 6 mm in diameter, which are placed in a Petri dish and sterilized in a hot air oven. After the sterilization, the discs were poured into the different concentration of broad spectrum antibiotics and into the prepared plant extract solutions and again kept under refrigeration for 24 hrs.

RESULTS AND DISCUSSION

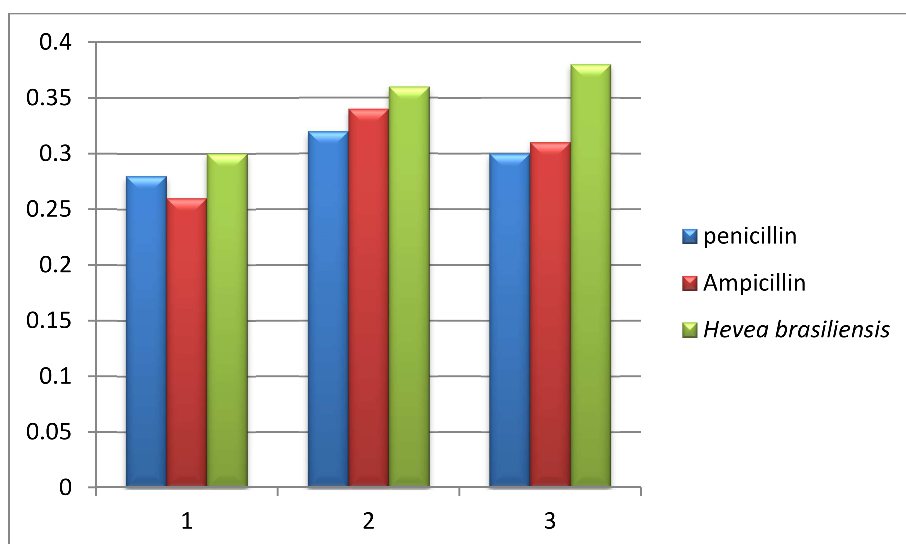
Extracts of plants and phytochemicals are getting more importance as potential sources for inhibiting different diseases during the recent decade. Ethnobotanical plants have a greater number of positive results than randomly selected plants.

Table 1. Preliminary Phytochemical constituents of Aqueous, Ethyl acetate, ethanol and Chloroformic extracts of *Hevea brasiliensis*

S.NO	Phytochemicals	Ethyl acetate	Ethanol	Chloroform	Aqueous
1.	Flavonoid	++	++	++	++
2.	Alkaloids	++	++	++	++
3.	Saponins	_ _	_ _	++	++
4.	Tanins	++	++	++	_ _
5.	Amino acid	++	++	++	_ _
6.	Protein	++	++	++	++
7.	Triterpenoids	_ _	_ _	_ _	_ _
8.	Reducing sugar	++	++	+ +	++
9.	Cardiac glycosides	++	++	+ +	_ _
10.	Anthroquinones	_ _	_ _	_ _	_ _
11.	Steroids	++	++	++	++

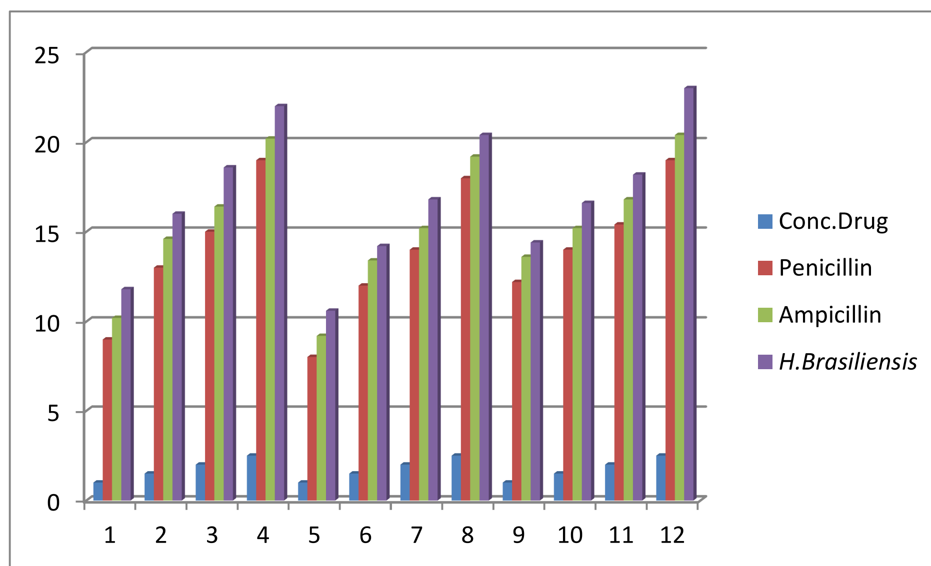
"++" - Positive. "--" - Negative.

Figure 1. Shows that the MIC value of penicillin and Ampicillin



. Group II, III and IV are compared with Group I.
. Group I, III and IV are compared with Group II.

Figure 2. Shows that the Zone of Inhibition (mm) of penicillin, Ampicillin and *H.Brasiliensis* against *E.coli, Pseudomonas Aeruginosa and Klebsila pneumonia*



. Group II, III and IV are compared with Group I.
. Group I, III and IV are compared with Group II.

Reading of Minimum Inhibition Concentration

Minimum inhibition concentration was expressed as the lowest dilution which inhibited growth judged by lack of turbidity in the tube, because very faint turbidity might be given by the inoculums itself. The inoculate tube was kept in the refrigerator overnight and was used as the standard for the determination of complete inhibition. The plant extracts were found to be effective against the three selected bacterial species [13]

Reading Zone of Inhibition and Interpreting Results

After 16 to 18 hrs of incubation each plate was examined. Once the resulting zones of inhibition came uniformly circular and in a confluent lawn of growth, the diameters of the zone of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the disc. Zones are measured to the nearest mm using a ruler, which was held on the back of the inverted Petri plate.[14].

- Clear inhibition zones indicated the presence of antimicrobial activity,
- Group II, III and IV are compared with Group I
- Group I, III and IV are compared with Group II.

Table 1. Showed that the phytochemical constituents of chloroformic Ethyl acetate and Water extracts of *Hevea brasiliensis*. The phytochemical screening of the crude extracts revealed the presence of Flavonoids, alkaloids, saponins, sugars, tannins, steroids and proteins were present. Saponins were present in chloroformic extract whereas, the Ethyl acetate extract shows negative result. In the case of flavonoids all extract showed positive result. The chloroform extracts shows the amino acids were present whereas water extracts showed negative result. The terpenoids were absent in all extracts. The reducing sugar were present in all extract. The cardiac glycosides absent in water extract and Anthroquinone were absent in all extracts.

The Phytochemical screening carried out with *Hevea brasiliensis* has revealed the presence of many secondary metabolites which interns contributed to its phytochemical pharmacological activities. The present study portrays that the phytochemical in a *Hevea brasiliensis* may contribute many significant ways for various studies in a truth full manner to the various activities of the plant in future.

Plants have been used for thousands of years to flavor and conserve food, to treat health disorders and to prevent diseases including epidemics. The knowledge of their healing properties has been transmitted over the centuries within and among human communities. Active compounds produced during secondary vegetal metabolism are

usually responsible for the biological properties of some plant species used throughout the globe for various purposes, including treatment of infectious diseases. Currently, data on the antimicrobial activity of numerous plants, so far considered empirical, have been scientifically confirmed, concomitantly with the increasing number of reports on pathogenic microorganisms resistant to antimicrobials. Products derived from plants may potentially control microbial growth in diverse situations and in the specific case of disease treatment, numerous studies have aimed to describe the chemical composition of these plant antimicrobials and the mechanisms involved in microbial growth inhibition, either separately or associated with conventional antimicrobials. Thus, in the present work, medicinal plants with emphasis on their antimicrobial properties are reviewed.

In the present study the results show that the extract from *H.brasiliensis* possess antimicrobial activities against *E.coli, Klebsila pneumoniae* and *Pseudomonas aeruginosa.* The extract compared favorably with the standard antibiotics Penicillin and Ampicillin. The plant extract showed more activity that broad spectrum antibiotic activities. The MIC of *H.Brasiliensis* were shown in table-1. The standard Penicillin and Ampicillin had MIC values varying between The E.coli value for Penicillin was 0.280 , for Ampicillin was 0.260 and for *H.Brasiliensis* it was 0.300 respectively. The *Pseudomonas aeruginosa value for* Penicillin , Ampicillin and *H.brasiliensis* was 0.320 , 0.340 , 0.360 Respectively. And for *Klebsila pneumoniae the values for* Penicillin was 0.300 Ampicillin was 0.310 and *H.brasiliensis* was 0.380 The results indicated that the extract of *H.brasiliensis* has stronger activity than that of standard antibiotics (Figure 1). Since ancient times, herbs and /or their essential oils have been known for their varying degrees of antimicrobial activities

Except for *E.coli, Klebsila pneumoniae* and *Pseudomonas aeruginosa*, *Enterococci* may be resistant to Ampicillin and Penicillin because of production of low affinity Penicillin binding protein (PBPs) or less commonly because of the production of β Lactamase. [11]. The disc diffusion test can accurately detect isolates with altered Penicillin binding proteins but it will not reliably detect β-lactamase producing strains. The rare β lactamase producing stains are detected best by using a direct, nitrocefin based β-lactamase test. Certain Penicillin, Ampicillin resistance *Enterococci* may possess high level of resistant (Penicillin -MIC>µg/ml or Ampicillin > 54 µg/ml). The disc diffusion test can not differentiate those with normal resistance from this high level resistance [12]. For, *Enterococcus* recovered from blood and CFS, the laboratory should consider determining the actual MIC from Penicillin or Ampicillin since *E . faceium* stains with normal lower level resistance (Penicillin MIC<54 µg/ml and Ampicillin <22 µg/ml) should be considered potentially susceptible to synergy with an ammioglycoside (in the absence of high level amminoglycoside resistance) whereas strains with higher level resistance may be resistant to such synergy

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