EVALUATION OF ANTIFUNGAL, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF AMPHINEURON EXTENSUS, HEDYOTIS DIFFUSA AND VITEX NEGUNDO

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Abstract: In the present study, the antifungal and antibacterial properties of three selected plants namely Amphineuron extensus, Hedyotis diffusa and Vitex negundo used in the preparation of rice beer starter culture cakes were evaluated against Aspergillus niger, Aspergillus flavus and E. coli. A. extensus showed the highest inhibition zone against A. niger at 20% concentration whereas H. diffusa showed the lowest MIC value of 1.25%. In case of A. flavus, V. negundo showed the highest Inhibition zone at 20% concentration and all the plant extracts exhibited MIC value of 5%. A. extensus showed the highest inhibition zone against E. coli followed by V. negundo and H. diffusa at 20% concentration. A. extensus also exhibited the lowest MIC value of 1.25% against E. coli. The antioxidant capacity of the methanol extracts was also estimated using DPPH method. V. negundo has the highest antioxidant capacity in comparison to other two plant extracts. These findings suggest the potential antimicrobial and antioxidant properties of the plant extracts and also their possible contribution to the overall medicinal and therapeutic benefits of rice beer.

Key words: Amphineuron extensus, Hedyotis diffusa, Vitex negundo, Antimicrobial, Antioxidant

INTRODUCTION

Rice beer is a traditional alcoholic beverage consumed on a daily basis by various ethnic tribes of North East India. This alcoholic beverage is believed to possess many medicinal and therapeutic properties possibly contributed by various indigenous herbs used in starter culture cake preparation. North East India is endowed with a variety of floral resources with high valued medicinal property. Research on these valuable resources is still in its infancy. The North East region of India is one of the ‘Biodiversity Hotspots’ of the world supporting about 50% of India’s biodiversity [1]. WWF in 2005 reported the North East India as the second richest centre in plant diversity. Traditionally many plant derived substances have been used to control human, animal as well as plant diseases. These plants possess the highest potential to be used as alternate medicine in modern science. Currently, development of antibiotic resistance in various microbial strains is a burning issue in medical science and the plant derived bioactive compounds can open new possibilities of combating this issue. Aspergillus niger is a major phyto-pathogen and responsible for black mould disease in certain fruits and vegetables [2]. It is also associated with Aspergillosis, otomycosis and production of Ochratoxin A [3,4]. Similarly Aspergillus flavus is the second most common agent of Aspergillosis after Aspergillus fumigatus. It also causes corneal, otomycotic, and nasoorbital infections in human. Many strains of A. flavus produce aflatoxin, a carcinogenic and acutely toxic compound that causes acute hepatitis, immunosuppression, and
hepatocellular carcinoma [5]. The virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. Literature also suggests that virulent strains are also responsible for mastitis, septicemia, hemolytic-uremic syndrome, peritonitis, and Gram-negative pneumonia [6].

There is a need to develop alternative agents for the control of pathogenic bacterial and fungal diseases in humans and plants. There is a good reason to support that the secondary metabolites of plants have evolved to protect them from attack by microbial pathogens. Hence, products from plants have great potential as sources of novel bioactive compounds for controlling pathogenic microorganisms. In general, plant-derived natural substances are considered as non-toxic and potentially effective against many pathogens. In recent years, interests have been generated in the development of safer antimicrobial agents such as plant-based essential oils and extracts to control many diseases. Historically, many plant oils and extracts have been reported to have antimicrobial properties. It is important to investigate scientifically those plants which have been used in traditional medicines as potential sources of novel antimicrobial compounds. Also, the resurgence of interest in natural control of pathogens and increasing consumer demand for effective, safe, natural products means that quantitative data on plant oils and extracts are required. In view of these, the present investigation was undertaken to screen the antimicrobial potency of three plant ethanol extracts against *Aspergillus niger*, *Aspergillus flavus* and *E. coli*. These plants are also used in the process of rice beer making.

**MATERIALS AND METHODS**

**Maintenance of pure culture of test microorganisms:** Pure cultures of *Aspergillus niger*, *Aspergillus flavus* and *E. coli* were maintained in Potato Dextrose Agar (PDA) and *E. coli* in Nutrient Agar (NA) slants at Assam Agricultural University, Assam. Stock cultures were sub cultured at regular interval.

**Preparation of plant extracts:** *Amphineuron extensus*, *Hedyotis diffusa* and *Vitex negundo* healthy plants were collected from Jorhat district of Assam. Selection of the plant samples was based on traditional uses in the preparation rice beer starter culture and easy availability. Identification of the plants was done on the basis of morphological characters. The individual plant samples were washed thoroughly, dried under shade and crushed into course powder and extracted with ethanol as per the standard protocol with little modification [7]. The plant material was then immerged in ethanol for 5 days with intermittent shaking. The extract was then filtered through Whatman filter paper No.1 and the filtrate was then dried at 40°C under reduced pressure in rotary evaporator (Heidolph Instruments GmbH & Co.KG, Germany) to obtain the crude extracts. Thus the stock extract of 100% concentration was obtained for each plant sample. The stock extracts were kept in airtight bottles for further studies.

Test extracts of desired concentrations (w/v) were prepared by dissolving the stock extracts in required amount of dimethyl sulfoxide (DMSO) and then adjusted the volume by adding distilled water and finally sterilized by filtering through millipore filter of 0.22 µm pore size (MILLEX® GP, Ireland). Test extracts so prepared were used for conducting *in vitro* antifungal and antibacterial efficacy.

**Agar well diffusion method for antimicrobial activity:** The crude extracts so obtained were evaluated for antifungal and antibacterial activity against the pathogens by Agar well diffusion method [8]. Test concentrations of the plant extracts were in the range of 20% - 0.625%. Broth inoculums were prepared by adding one loop full of pure cultures of the test fungi and bacteria in conical flask containing 50 ml of potato dextrose broth and nutrient broth and then incubated at 28 ± 2°C and 35 ± 2°C for 48 hrs respectively. Agar plates of 80 mm were inoculated with 100 μl broth cultures of the test pathogens and two wells of 7 mm diameter each were made with a sterile cork borer. The wells were filled with 150 μl test extracts and allowed to diffuse at room temperature for 2 hours. Then the plates were incubated for 48 hours. The antimicrobial activity of the extracts was determined by measuring the diameter of the inhibition zone around the well. DMSO was used as control while bavistin (0.01%) and streptomycin (10μg) were used as standards for fungal and bacterial pathogens respectively.

**Determination of minimum inhibitory concentration (MIC):** To determine the MIC values of different plant extracts, two-fold serial dilutions of 20, 10, 5, 2.5, 1.25 and 0.625 mg/ml of the extracts were prepared and tested by Agar well diffusion
Percentage inhibition: Percentage of inhibition was calculated according to the following formula [9].

\[
\text{% Inhibition} = \frac{\text{Inhibition zone in mm}}{\text{Control}*} \times 100
\]

*Growth zone is equal to plate diameter i.e., 80mm as growth occurs all over the agar plate.

Activity index determination: The activity index of the extracts was determined by the following formula [10].

\[
\text{Activity index} = \frac{\text{Inhibition zone of extracts}}{\text{Inhibition zone of standard}}
\]

Total antioxidant capacity: Total antioxidant capacity of the plant extracts was estimated on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity by modified method of Khalaf et al. [11]. The diluted working solutions of the test extracts were prepared in methanol. Ascorbic acid was used as standard. 100μl of aliquot of the extracts were taken in a test tubes and 2.9ml of DPPH reagent (0.5mM in 99.5% of methanol) was added to each tubes and vortexed vigorously. Then the tubes were incubated in dark for 30min at room temperature. The discoloration of DPPH is measured against blank at 517nm. DPPH methanolic solution was used as blank. Percentage of inhibition was calculated as follows:

\[
\text{% Inhibition (Antiradical Activity)} = \frac{A_B - A_A}{A_B} \times 100
\]

Where \(A_B\) is the absorbance of the control reaction (includes all reagents excluding the test compound), and \(A_A\) is the absorbance of the test sample. The concentration of extract providing 50% inhibition (IC50) was calculated from the graph plotting inhibition percentage against extract concentration. IC50 is the concentration of the extract needed to inhibit half of the present free radicals.

RESULTS AND DISCUSSION

Antimicrobial activity: Ethanol extracts of Amphineuron extensus, Hedyotis diffusa and Vitex negundo exhibit significant antifungal and antibacterial properties against A. niger, A. flavus and E.coli (Tables 1, 2). Highest inhibition zone of 24 mm was observed against A. niger when A. extensus at 20% concentration was used followed by H. diffusa and V. negundo showing inhibition zones of 22 mm and 19 mm respectively. Both A. extensus and V. negundo showed the MIC values of 2.5%, whereas H. diffusa was found to have MIC value of 1.25% against A. niger. Interestingly A. extensus possessed the highest Percentage of Inhibition and activity Index determination values of 30 and 1.33 respectively against A. niger. Therefore, the antifungal activity exhibited by all the three plant ethanol extracts at 20% concentration was much higher than the standard (bavistin 0.01%) which showed only 18 mm of inhibition zone. A. flavus and V. negundo showed the highest inhibition zone of 18 mm followed by A. extensus and H. diffusa showing 17 mm and 15 mm respectively. Similarly, V. negundo was found to have the highest Percentage of Inhibition and Activity Index values of 22.5 and 0.82 respectively. All the plant extracts exhibited MIC value of 5% against A. flavus. An inhibition zone 22mm was observed against A. flavus in case of standard (bavistin 0.01%).

A. extensus showed the highest inhibition zone 26.5 mm against E. coli followed by V. negundo and H. diffusa showing 24mm and 20mm respectively at 20% concentration. Similarly, A. extensus was also found to possess the highest Percentage of Inhibition and Activity Index values of 33.12 and 2.038 respectively at 20% concentration. A. extensus also showed the lowest MIC value of 1.25%, whereas both V. negundo and H. diffusa showed MIC values of 5%. In case of standard (streptomycin), an inhibition zone of 13 mm was observed. This indicates that the plant extracts possess much higher activity at 20% and 10% concentrations in comparison to the standard.

Antiradical activity: Results obtained in the study showed that the IC50 of methanol extract of V. negundo (18.2 μg/ml) was significantly lower than that of both A. extensus (23.6 μg/ml) and H. diffusa.
Table 1: *In vitro* antifungal and antibacterial activity of plant ethanol extracts at different concentrations. *Values are mean of three replicates.

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>%I**/ AI***</th>
<th>A. niger</th>
<th>A. flavus</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphineuron extensus</td>
<td>A. niger</td>
<td>30</td>
<td>21.25</td>
<td>18.75</td>
</tr>
<tr>
<td>Hedyotis diffusa</td>
<td>A. niger</td>
<td>27.5</td>
<td>18.75</td>
<td>14.2</td>
</tr>
<tr>
<td>Vitex negundo</td>
<td>A. niger</td>
<td>23.75</td>
<td>22.5</td>
<td>14.2</td>
</tr>
</tbody>
</table>

Table 2: % Inhibition and Activity Index of the plant extracts. **Percentage Inhibition at 20% concentration. ***Activity Index at 20% concentration.
extracts (34.5 μg/ml) (Fig. 1). This indicates that the *V. negundo* methanol extract has the highest antioxidant capacity in comparison to other two plant extracts. An antioxidant inhibits the oxidation of lipid or other molecules providing protection against reactive oxygen species [12]. Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing [13,14]. Antioxidant activity of several plant extracts and different classes of phytochemicals have been found to be quite prominent [15]. Antioxidant activity of plant materials are dependent on the nature of the different solvent extraction system [12]. Water, methanol, mixture of water-methanol, acetone have been widely used to extract antioxidant compounds from various plants and plant-based foods [16]. These compounds are known to inhibit the oxidative mechanisms which are responsible for many disorders and diseases in humans such as infections, diabetes, arthritis, cardiovascular diseases, cancer, Alzheimer’s diseases, AIDS etc. [17].

Methanol extract of *V. negundo* was earlier found to possess significantly higher antioxidant activity in terms of DPPH free radical scavenging activity. This methanol extract also contained high amounts of bioactive compounds including total phenolic compounds, epicatechin, quercetin, catechin and myriceti low amount of tocopherol, β-carotene and lycopene [18]. Similarly, in another past research finding, the antioxidant activity of methanol extract of *V. negundo* was 23.21 mg/100 of ascorbic acid equivalent antioxidant capacity and presence of phytochemicals like 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-(RT:6.17), Phytol (RT:19.67) and vitamin E (RT : 25.11) were also detected [19]. Whereas the percolate extract of *V. negundo* exhibited a strong scavenging activity with EC50 value of 2.85 μg/ml against ascorbic acid as control which exhibited EC50 value of 18.70 μg/ml [20]. *H. diffusa* also showed good antioxidant properties and contained high amount of phenolic compounds like phenolic acids (p-coumaric acid) and flavonols (kaempferol glycosides) [21]. It has been used for the treatment of hepatitis, tonsillitis, sore throat, appendicitis, urethral infection and malignant tumors of the liver, lung and stomach [22]. These observations support our current findings and also states that the antioxidant activity is mainly due to the presence of the various phenolic compounds in the extracts.

Our results also suggest the contribution of these plants to the overall therapeutic and medicinal properties of rice beer. A variety of such plants are used in the preparation of rice beer starter culture cakes. Further investigation is needed to prove the possible medicinal and therapeutic properties of rice beer produced in North East India.

The ethanol extracts are mixtures of multiple components, generally a mixture of active and inactive compounds and therefore higher doses are expected as compared to commercial antibiotics and fungicides. With more refined and solitary compounds, an effective antimicrobial activity from small amount may be obtained. The bioactive compounds isolated from these plants have tremendous potential for developing antibacterial and antifungal formulations to cure various human and plant diseases. These compounds are relatively safe and possess the potential to fight against various antibiotic resistant microbial strains. The plant derived bioactive molecules can also be used in the effective management of plant diseases to maintain high level of bio-safety and non-adverse effects on the environment unlike other carcinogenic chemical fungicides.

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**Abbreviations used:** IC= Inhibition Concentration; IC50 = 50% Inhibition; MIC= Minimum Inhibitory Concentration; AI = Activity Index; DMSO = dimethyl sulphoxide; % I =Percentage Inhibition.

**REFERENCES**


