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EVALUATION OF ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF THE BARK OF *HOLARRHENA PUBESCENS*, ITS FRACTIONS AND CONESSINE

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ABSTRACT

The present study was carried out to investigate the antioxidant effect of the methanolic extract, its alkaloidal and non-alkaloidal fractions along with petroleum ether soluble, ether soluble and ethyl acetate soluble sub-fractions of non-alkaloidal part of the bark of *Holarrhena pubescens*. The pure compound conessine was also tested. The activity was determined by using DPPH free radical scavenging assay at 500 µg/mL for the extract and the fractions, and at 200 µg/mL for conessine and the standard (ascorbic acid). The non-alkaloidal fraction was found to be more active (63% inhibition; EC_{50} = 250 µg / mL) than the alkaloidal fraction (16% inhibition) and its polar ethyl acetate soluble fraction was found to be most active (70% inhibition; EC_{50} = 250 µg / mL). Conessine was non-active at the concentration used.

Keywords: *Holarrhena pubescens,* Apocynaceae, Bark, Antioxidant and DPPH free radical scavenging assay.

INTRODUCTION

Holarrhena pubescens (Buch. Ham) (Synonym: *H. antidysenterica*)[1] belongs to the family Apocynaceae. It is a typical Indian medicinal tree 30-40 ft. high and upto 4 ft. in girth. The tree is common in the forest of India and Pakistan, indigenous to the tropical Himalaya and Assam. The stem bark of this plant, commercially known as "kurchi" has, antidiarrhoeal, antidysenteric, anthelmintic, stomachic, digestive, astringent and tonic properties. It is also usefull in piles, toothache, chest infections, diuresis, skin and spleen diseases. The seeds are effective in diarrhea, jaundice, and stone in bladder. The main alkaloid conessine has been used in amoebic dysentery and vaginitis. It also retards the growth of tubercle bacilli[2-6]. Antibacterial activity of *H. pubescens* and other *Holarrhena* species were reported against a few organisms[7,8]. More recently S. Surverwaran and co-workers [9] reported antioxidant activity in the fruit extract of *H. antidysenterica*. The chemical constituents of various parts have been investigated by many groups. Some recently reported compounds include alkaloids holamine, kurchamine, holaphyllidine, holaromine, mitiphylline, holadysenterine[10] and non-alkaloidal constituents kurchinin[11], kurchinicin[12], holarrhenol[13] etc. Reactive oxygen species (ROS) play an important role in the host defence mechanism against microorganisms, but an increased production of ROS causes a variety of diseases such as cancer, inflammation, neurodegeneration, Parkinson's disease, atherosclerosis, and pre-mature aging[14-19]. Oxidative stress occurs in the living organisms as a result of an increase in oxidative metabolism, which produces a number of ROS. To avoid oxidative stress, antioxidants can play an important role in giving beneficial effects[20]. For evaluating antioxidant

activity, 1, I-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay is a simple and very useful as a primary screening system.[21] DPPH is a stable free radical possessing an odd electron. It gives a strong absorption band at 517 nm (deep violet color). As this electron becomes paired off, the absorption decreases stoichiometrically with respect to the number of electrons taken up. This change in absorbance has been widely used to test the free radical scavenging activity of natural products.[21] In the present work the methanolic extract of *H. pubescens*, its fractions and the main steroidal alkaloid, conessine, were evaluated for their antioxidant activity by DPPH scavenging assay.

MATERIALS AND METHODS

Plant Material: The bark of *H. pubescens* (*H. antidysenterica*) was supplied by the courtesy of Hamdard Foundation Pakistan (Pvt.) Ltd. It was identified by Miss Ashreen Jahan, a botanist at Hamdard Foundation Pakistan Ltd.

Extraction and Isolation: Uncrushed bark (10 kg) of *Holarrhena pubescens (H. antidysenterica)* was macerated over night with 10% methanolic NaOH, and repeatedly percolated with MeOH for 48 h (five times) at room temperature. The extract was neutralized with 30% aqueous HOAc and freed of the solvent under reduced pressure. The syrupy concentrate (**HP**) obtained from the combined extracts was acidified with 10% aqueous HOAc and extracted out with EtOAc. The aqueous phase was basified with 20% NH_4OH and again shaken out with EtOAc. The moist EtOAc phase was treated with a vigorous stream of CO_2. The precipitate containing the carbonate bases was filtered and the filtrate was dried over anhydrous Na_2SO_4 and freed of the solvent under reduced pressure. The residue (HP-B; 20 g) from the filtrate was divided into petroleum ether soluble and petroleum ether insoluble fractions. The petroleum ether soluble fraction yielded conessine (9 g) according to the reported isolation procedure[22]. The structure of conessine was established with the help of exhaustive NMR studies and comparison of spectral data with reported values.[23] The main non-alkaloidal ethyl acetate phase was washed, dried (Na_2SO_4) and freed of the solvent under reduced pressure. The residue (HP-EA) thus obtained was divided into petroleum ether soluble (HP-N-PE) and pet-ether insoluble fractions. The petroleum ether insoluble fraction was further divided into ether soluble (HP-N-E) and ether insoluble portions. The ether insoluble fraction was dissolved in ethyl acetate (HP-N-EA) and the ethyl acetate insoluble darkish minor material was discarded. The methanolic extract (HP), its fractions HP-B, HP-EA, HP-N-PE, HP-N-E and HP-N-EA, as well as conessine were tested for their antioxidant activity (Table 1).

Determination of DPPH radical scavenging activity: The antioxidant activity was determined by the method described by Lee and his co-workers [21]. A solution of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (333 μM) was prepared in ethanol. Test samples were prepared by dissolving them in dimethylsulfoxide (DMSO). Reaction mixtures containing 10 μL of test samples and 90 μL of $DPPH^{\cdot}$ solution was added in 96 well microtiter plates (final concentration of test sample was 500 μg/mL for fractions and 200 μg/mL for pure compound and final concentration of DPPH in the well is 300 μM). Plates were incubated at 37°C for 30 minutes. Absorbance was measured at 515 nm using spectrophotometer. Percent inhibition by sample treatment was determined by comparison with a DMSO treated control group.

$$\% \text{ Inhibition} = \frac{(\text{Absorbance of the control} - \text{Absorbance of test sample})}{\text{Absorbance of the control}} \times 100$$

Ascorbic acid was used as positive control. EC_{50} value calculated denotes the concentration (in μg/mL) of sample required to scavange 50% of DPPH free radicals.

RESULTS AND DISCUSSION

In the present studies, the antioxidant activity of the methanolic extract (HP), its fractions (HP-B, HP-EA, HP-N-PE, HP-N-E and HP-N-EA) and conessine was determined. The non-alkaloidal fraction (HP-EA) which showed 63% inhibition of DPPH at 500 μg/ml concentration was divided into petroleum ether soluble (HP-N-PE), ether soluble (HP-N-E) and ethyl acetate soluble (HP-N-EA) fractions. The free radical scavenging activities of these fractions were in the order: HP-N-EA > HP-N-E > HP-N-PE (Table 1). These results suggest that the ethyl acetate soluble fraction contained the strongest free radical scavenging compounds. However, neither the fractions nor the pure compound conessine was as effective as a DPPH free radical scavenger as the positive control ascorbic acid (EC_{50} = 9.4 μg/ml). The results indicate that the activity increases as the polarity increases and the non-alkaloidal ethyl acetate soluble fraction HP-N-EA was the most effective radical scavenger. It exhibited 70% inhibition of DPPH (EC_{50} 250 μg/ml). The crude extract (HP), the alkaloidal fraction (HP-B) and conessine were very weak radical scavengers showing 22%, 16% and 12% inhibition of DPPH respectively (Table-1).

CONCLUSION

This is the first report of the antioxidant activity of *H. pubescence* growing in Indo-Pakistan. The results of antioxidant activity indicated that the non-alkaloidal fraction is more active (63%; EC_{50}=250µg/mL) than the alkaloidal fraction (16% inhibition) and its polar ethyl acetate soluble fraction was found to be most active (70 %; EC_{50}=250µg/mL) than ether soluble (52 %; EC_{50}=500µg/mL) and petroleum ether soluble (38%) fractions. Conessine was isolated in considerable quantity.

Table 1: *In Vitro* Antioxidant Activity of Methanolic Extract (HP), Its Fractions and Conessine by DPPH free radical scavenging assay

Sample	**% Inhibition (500 µg/mL)**	**EC_{50} (µg/mL)**
HP	22	>500
HP-B	16	>500
HP-EA	63	250
HP-N-EA	70	250
HP-N-E	52	500
HP-N-PE	38	>500
Conessine	12*	>200
Ascorbic acid (Positive control)	87*	9.4

**At 200 µg/ml concentration*

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