DOI: 10.7860/JCDR/2014/7803.4863

Original Article

Pharmacology Section

Evaluation of Antioxidative and Antidiabetic Activity of Bark of *Holarrhena Pubescens Wall*

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ABSTRACT

Objective: The objectives of the study are to screen out various phytochemicals and to evaluate the antioxidant and antidiabetic potential of the stem bark of *Holarrhena pubescens Wall* (*Holarrhena antidysenterica*).

Materials and Methods: The antioxidant activity was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity where ascorbic acid was taken as positive control. The antioxidant property was later exploited and the methanolic extract of plant was tested for antihyperglycemic activity in glucose overloaded hyperglycemic mice. The extract was tested for its hypoglycemic activity at two-dose levels, 250 and 500 mg/kg respectively where Glipizide 5 mg/kg was taken as standard reference drug. All results are presented as mean ± SD (Standard Deviation). Significant differences between experimental groups were determined by Student's t-test.

Results: The methanolic and water extract showed strong antioxidant activity with inhibition of more than 90% DPPH free radicals at the concentration of 100µg/mL. The hypoglycemic activity of methanolic extract on glucose tolerance test were significant ($p < 0.05$) for the effects of 500 mg/kg after 120 min of treatment and ($p < 0.01$) for 250 mg/kg of extract after half hour of treatment compared to control.

Conclusion: The presence of flavonoides, phenolic compounds suggested that they may be partially responsible for antioxidant and antidiabetic activity.

Keywords: Antioxidant, Antidiabetic, DPPH, EDTA, *Holarrhena pubescens*, *Holarrhena antidysenterica*

INTRODUCTION

Diabetes mellitus(DM), which is associated with oxidative damage, has a significant impact on health, quality of life, and life expectancy [1]. DM, characterized as genetically based predisposition and dietary indiscretion [2] is ranked seventh among the leading causes of death and third when it's fatal complications are taken into account [3,4]. There is increasing evidence that complications related to diabetes like retinopathy, atherosclerotic vascular disease are associated with oxidative stress induced by the generation of free radicals [5]. Implication of oxidative stress in the pathogenesis of diabetes mellitus is also suggested due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes and formation of lipid peroxides [6]. High phenolic and antioxidants containing plants are found to have higher antihyperglycemic activity in diabetes [7], so many plants, herbs and spices are studied since time immemorial to evaluate the antioxidant and antidiabetic potential.

H pubescens is a flowering plant which belongs to the apocynaceae family and its bark is commonly used in diarrhea, fever, piles, jaundice, stone in bladders, amoebic dysentery and vaginitis [8–10].

Some recently reported compounds include alkaloids holamine, kurchamine, holaphyllidine, holaromine, mitiphylline, holadysenterine and non-alkaloidal constituents kurchinin, kurchinicin, holarrheno [11]. The aqueous extract of the seeds of this plant has been found to be effective in diabetes [12]. The bark powder of this plant is commonly used as an effective drug in several formulations against diabetes in several parts of Nepal however no scientific study has been documented in literature.

MATERIALS AND METHODS

Collection of plant material

The bark of *H pubescens* was collected from the place Sat Mane, Khudi within the Lekhnath Municipality-12 which is near to Pokhara University. Collection was done during the month of June/July, 2010. Then the barks were thoroughly washed with water to remove the latex and other dirt. The lichens present on the surface of the barks were removed by scrapping.

Extraction of plant material

Dried and powdered plant material (151.1 g) was successively reflux extracted with hexane, ethylacetate (EtOAc), methanol (MeOH) and water for 2hrs each respectively. The obtained extracts were evaporated in vacuum to give residues and their percentage yields were determined.

Phytochemical screening

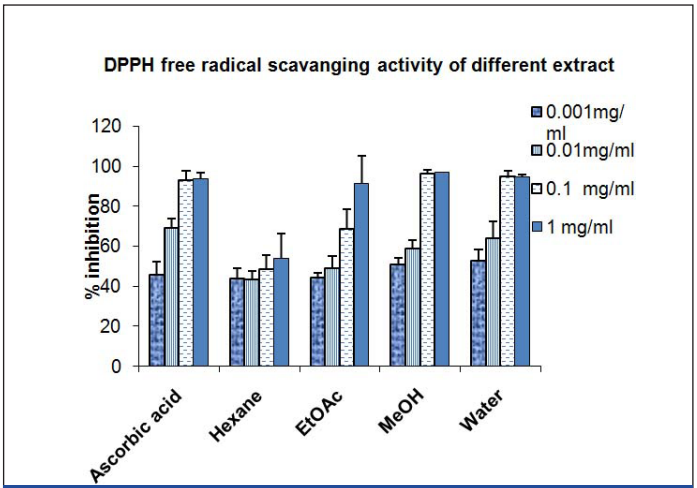
Phytochemical screening was carried out for hexane, methanol and water soluble fractions as per the standard methods [13].

Animals and treatment

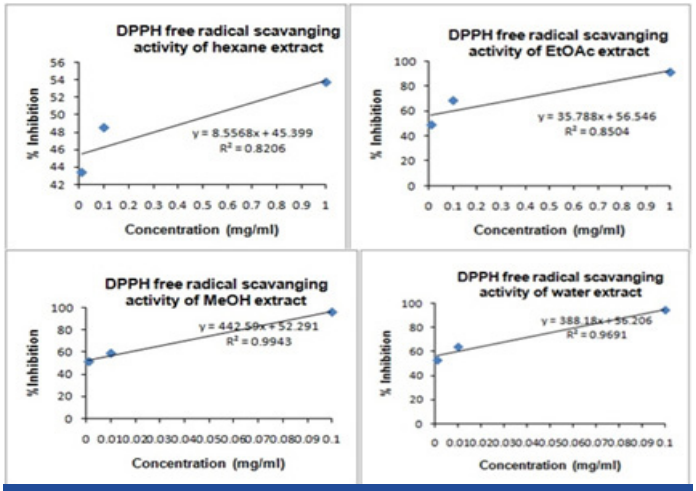
Healthy male Swiss Alabino mice (30-35 g) with no prior drug treatment were used for the present studies. The animals were fed with standard pellet diet and water ad libitum. The animals were acclimatized to laboratory hygienic conditions for a period of a week before starting the experiment. Animal study was performed in the Pharmacology lab, School of Health and Allied Sciences, Pokhara University, NEPAL after approval from the Institutional Animal Ethics Committee.

Evaluation of anti-oxidant activity DPPH radical-scavenging activity test

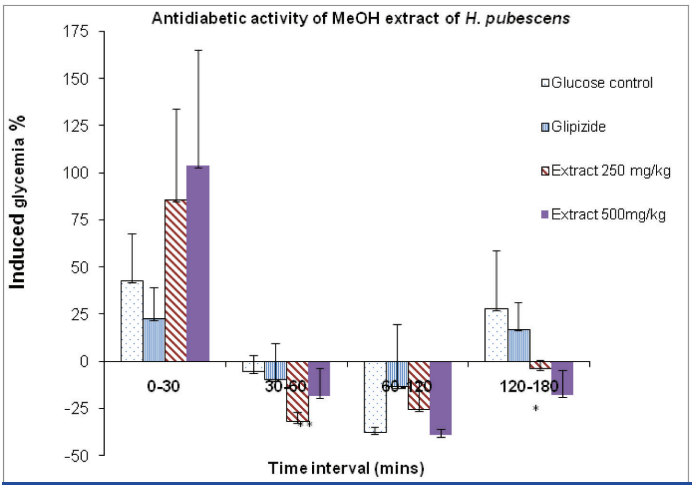
Antioxidant activity was measured by DPPH radical-scavenging method [14]. Solutions of concentrations 0.001 mg/mL, 0.01 mg/mL, 0.1 mg/mL and 1 mg/mL were prepared for each extract. Briefly, 2mL of 0.1 mM DPPH methanolic solution was added to 2 mL of each extract of *H. pubescens* at different concentrations (0.001 – 1 mg/mL). The mixture was shaken vigorously then left to stand at room temperature. Ascorbic acid solutions of same concentrations



[Table/Fig-1]: Percentage Inhibition of DPPH free radical by extracts/ascorbic acid at 520 nm
Data are expressed as mean value ± SD (n=3).DPPH Radical Scavenging activity (%) = (A-B)/A* 100 %, Where A is the control absorbance and B is the sample absorbance. Control is the test solution without sample. Ascorbic acid was used as the positive control



[Table/Fig-2]: Graphical representation of DPPH free radical scavenging activity of Hexane, EtOAc, MeOH and Water extracts



[Table/Fig-3]: Percentage inhibition of glucose by different extract at different time interval
Each value represents the mean ± SD (n = 3). * p≤0.05, ** p< 0.01 vs control

0.001 mg/mL – 1 mg/mL were prepared and used as positive control for the radical - scavenging activity test. Thirty minutes later, the absorbance was measured at 520nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

The capability to scavenge the DPPH free radical was calculated using the following equation:

Percentage of radical scavenging activity = [(Abscontrol – Abssample) / Abscontrol] × 100%

Where, Abscontrol = Absorbance of DPPH solution

Abssample = Absorbance in presence of extracts or ascorbic acid solutions.

Determination of antidiabetic activity Oral glucose tolerance test (OGTT)

Antidiabetic activity was studied in glucose overloaded hyperglycemic mice. The animals were divided into four groups (n=8). Group I animals served as control and were treated with 3 g/kg glucose. Group II animals were treated with 5 mg/kg of standard drug Glipizide and the remaining two groups were treated with 250 mg/kg and 500 mg/kg (p.o) of methanolic extract of *H. pubescens*. Zero hour blood sugar level was determined from overnight fasted animals. After 30 min of drug treatment (p.o), the animals were fed with glucose (3 g/kg) and blood glucose was determined after 0.5, 1,2 and 3 h of the glucose load. Blood glucose level was estimated by one touch glucometer. The blood collection was done byTail vein method [15–17].

STATISTICAL ANALYSIS

All results are presented as mean ± S.D (Standard Deviation). Significant differences between experimental groups were determined by Student's t-test. The level of tested significance was $p<0.05$ and each experiment was performed two or three times.

RESULTS

Preliminary phytochemical screening

The percentage yield for hexane, ethyl acetate, and methanol was found to be 5.91, 1.653 and 15.1568% w/w respectively. Hexane soluble fraction contains alkaloids, resins, tannins, fats and oils. Methanol extract contain alkaloids, phenols and water soluble fraction contained alkaloids, phenol, diterpene, tannins and fats and fixed oils.

Antioxidant sensitivity test DPPH free radical scavenging method

The positive DPPH free radical scavenging suggests that the compounds are electron donors. The scavenging effects of different extract at different concentration generally increased with the polarity of solvent. The scavenging activity of positive control; Vitamin C increased with increasing concentrations and the maximum inhibition of DPPH radical was more than 90 % at 0.1 mg/mL. Among the various extracts, EtOAc and MeOH showed DPPH free radical scavenging activity as concentration dependent manner and maximum inhibition was exhibited by methanol extract of about 96% at 0.1 mg/mL. Among other extract, water extract showed similar activity to that of methanol whereas hexane extract showed weak DPPH radical scavenging activity [Table/Fig-1]. The effective concentration (EC50) value for hexane, EtOAc, MeOH and water extract was found to be 0.537 mg/mL, 0.183 mg/mL, 0.005 mg/mL and 0.015 mg/mL respectively [Table/Fig-2].

Antidiabetic activity test

250 mg/kg and 500 mg/kg (p.o) of methanolic extract of *H. pubescens* had no toxic effect on mice. After 30min of the glucose (5 mg/kg) load, the blood glucose level was measured and it was found that blood glucose level in all the group of mice was raised to maximum point and after that the glucose level started to decline. From 30 minutes to 60min, the blood glucose level of methanolic extract (250mg/kg) administrated mice was significantly decreased whereas there is no significant difference in other groups. At 120min of glucose load, there is no significant difference however at 10 min

of glucose load, methanolic extract (500 mg/mL) administrated mice showed significant decrease in the blood glucose level compared to control group [Table/Fig-3].

DISCUSSION

The present study was conducted to evaluate the antioxidant and antidiabetic activity of *H. pubescens*. The phytochemical screening revealed the presence of carbohydrates, alkaloids, saponins, resins, phenols, proteins and amino acids in hexane, methanol and water soluble fractions.

In DPPH free radical scavenging study, almost all the extract showed the positive result. Among them MeOH and water extract showed highest radical scavenging activity [Table/Fig-1]. This may be due to the presence of polar compounds like phenols, polyphenols, flavonoids etc which are soluble in water and methanol [18]. Bibave et al., [19] have also documented free radical scavenging activity (Antioxidant) with aqueous and methanolic extract of *H. pubescens* (200 – 1000 ug/ml) in DPPH assay with ascorbic acid as standard references [19].

The extracts might contain antioxidants of natural origin that counter the deleterious free radicals which abstract H+ from hydrogen donors (e.g. phenol) and accept electron from electron rich species causing oxidative stress in aerobic mechanism. As a conclusion, these antioxidants from *H. pubescens* may be useful in scavenging free radicals and treating diseases related to free radical reactions.

In diabetes like disease, oxidative stress has been found to be mainly due to an increased production of oxygen free radicals and sharp reduction of antioxidant defences. Therefore, the antioxidant activity along with antihyperglycemic effects of *H. pubescens* can reduce the oxidative stress and lower blood glucose.

Experimental induction of hyperglycemia by intragastric ingestion of glucose resulted in a 1.5 to 2-fold increase in plasma glucose levels of the fasted mice. The methanolic extract at 250 mg/kg and 500 mg/kg produced significant ($p< 0.05$) hypoglycemic effect in the fasted normal mice after 1h and 3h of oral administration, when compared with normal control group [Table/Fig-3]. The results indicated that in fasted mice there was significant increase in blood glucose level after 30min of administration of methanolic extract of *H. pubescens* at dose of both 500 mg/kg and 250 mg/kg. This indicates the fact that the extract may consist of some traces of reducing sugar responsible for increment of blood glucose level initially in first half hour and also the antidiabetic components responsible for decrement of blood glucose level till third hour. The maximum hypoglycaemic activity of the plant was observed with the methanol extract at the dose of 500 mg/kg b.w. with a reduced percentage of blood glucose of 39.7% during 1-2 h of treatment compared to 13% obtained for the standard drug Glibenclamide at the same time.

Our finding of antihyperglycemic activity of methanolic extract of *H. pubescens* bark is supported and are similar to findings documented by Bandawane et al.,[20] and Bibave et al., [19]. Bandawane et al., documented that methanolic extract of *H. pubescens* bark (200, 400 mg/kg) significantly ($p<0.01$) reduced the increase in blood glucose levels at 60, 90, and 120 min in glucose loaded rats when compared with diabetic control rats [20]. Similar findings of antihyperglycemic effect were documented by Bibave et al., with aqueous and methanolic extract of *H. pubescens* bark on normal rats. After 90min of glucose administration, significant decrease in blood glucose was observed with 200 mg/kg and 400 mg/kg respectively [19].

OGTT reveals that the hypoglycaemic effect is seen by the end of the first hour and this level is maintained up to 3h. It is possible that the active principle in the total extract takes time to reach and act on the target tissues, eliciting a response to maintain the hypoglycaemic level, though the exact mechanism of action cannot be ascertained through these investigations.

In many studies alkaloids have been found to have hypoglycemic activity by inhibiting glucose transport through the intestinal epithelium. Hypoglycemic plants are found to contain polysaccharides, polyphenols and the various experimental results indicate that these compounds increase the levels of serum insulin, reduce the blood glucose levels and improve tolerance of glucose [17]. In the recent years, the positive antioxidant effects of some antidiabetic herbal products are also established [21]. As a conclusion, it could be speculated that the observed antihyperglycemic activity of *H pubescens* barks might be related to the presence of alkaloids, polysaccharides and saponins. However, further study is needed to elucidate the active constituent which has an antidiabetic effect.

CONCLUSION

Considering the phytochemical screening and the DPPH radical scavenging activity as indices of antioxidant activity of the extract, the findings revealed the potential of the extract as a source for natural antioxidants. The traditional uses of *H pubescens* to treat diabetes are supported from this study, suggesting a need to isolate and evaluate active constituents responsible for the exhibited biological activity.

ACKNOWLEDGEMENT

The authors are thankful to the management of Pokhara University, Pokhara, Nepal for giving the necessary permission, support to conduct the animal study and for providing necessary research facilities.

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FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Feb 03, 2014**
Date of Peer Review: **Apr 05, 2014**
Date of Acceptance: **Jun 26, 2014**
Date of Publishing: **Sep 20, 2014**