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Research Article

ISOLATION, CHARACTERIZATION OF PHYTOCONSTITUENTS AND PHARMACOLOGICAL SCREENING OF *IPOMOEA STAPHYLINA*

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ABSTRACT

The present study evaluates the methanolic and hydro alcoholic extracts of whole plant, stem, root, flower, leaves of *Ipomoea staphylina* for their *in-vitro* antioxidant activity (by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and superoxide radical scavenging method), *in-vitro* antidiabetic activity (by α-glucosidase and α-amylase inhibitory method) and *in-vitro* anti-inflammatory activity (by 5- lipoxygenase inhibitory method) . DPPH radical scavenging activity of stem hydro alcoholic and leaf methanolic extract of *Ipomoea staphylina* is found to be more potent as compared to other extracts with IC_{50} values 17.67 μg/ml, 32.77μg/ml respectively. Superoxide radical scavenging activity of stem hydro alcoholic and leaf hydro alcoholic extracts of *Ipomoea staphylina* is also found to be potent compared to other extracts with IC_{50} values 15.54 μg/ml, 16.02 μg/ml respectively. So methanolic extract of *Ipomoea staphylina* leaves was subjected to column chromatography and two compounds namely Sitosteryl-3-O-β-D-glucoside (1) and chiro deoxy inositol (2) were isolated and characterized by various spectroscopic and chromatographic techniques. These two constituents are first reported in *Ipomoea staphylina*.

Keywords: *Ipomoea staphylina*, Antioxidant activity, Anti-inflammatory activity, Anti-diabetic activity.

INTRODUCTION

Diabetes is a metabolic disorder associated with an increased risk of developing vascular and other health complications. Oxidative stress and inflammation are the major pathogenetic mechanisms considered to be implicated in diabetes[1-2]. Subjects with type 2 diabetes have been shown to have increased levels of lipid peroxidation, oxidative damage to DNA and protein oxidation[1], presumably caused by an over production of free radicals and a decreased antioxidative defence. Enhanced production of free radicals is related to hyperglycaemia, insulin resistance and hyperinsulinaemia[3]. High levels of glucose lead to an increased production of free radicals via different mechanisms such as glucose autooxidation and formation of advanced glycation end products[4].

Besides oxidative stress, inflammation is also implicated in the development of complications in type 2 diabetes[2]. Cyclo-oxygenase catalyzed PG formation, and subsequently low-grade inflammation is suggested to be an early event in the development of type-2 diabetes which is further linked to oxidative stress[5]. Elevated levels of high sensitivity C-reactive protein (hsCRP) and IL-6 are seen in subjects with type-2 diabetes[6], and also associated with an increased risk for developing the other diseases in future[7]. However inflammation and oxidative stress are related to glycaemic control is still not fully clarified.

Ipomoea staphylina is commonly found on hedges and bushes in the forests and waste lands. It is a perennial, woody and glabrous shrub with pink flowers. Traditionally *Ipomoea staphylina* is used for respiratory disorders[8-11]. Traditionally genus *Ipomoea* is used as purgative, dyspepsia, anthelmintic, bronchitis[12].

So in present study *Ipomoea staphylina* is evaluated for *in vitro* antioxidant, antidiabetic and anti-inflammatory pharmacological activities.

MATERIALS AND METHODS

Chemicals and instruments - ^{1}H- and ^{13}C-NMR, H-H correlation spectroscopy (COSY), NOE, and HMBC spectra were recorded on an Bruker Avance 400 MHz spectrometer in $CDCl_3$, unless otherwise stated. Chemical shifts are shown in δ values (ppm) with tetramethylsilane (TMS) as an internal reference. Mass spectra were recorded on Agillant 1100 Series LC-MS spectrometer. IR spectra were recorded on a Perkin Elener Spectrum BX, FT-IR System. TLC analyses were performed on Kieselgel 60 F_{254} (Merck) plates (silica gel; 0.25 mm layer thickness); compounds were visualized by dipping plates into 20% (v/v) H_2SO_4 reagent and then heated at 110 ^{o}C for 5-10 min. Silica gel (Merck 60A; 60-120 mesh) was used for column chromatography. All chemicals were procured from Merck India Pvt. Ltd.

Plant Material

Whole plant, stem, root, flower and leaves of *Ipomoea staphylina* were collected from Mailavaram, Krishna district, AP, India in January 2010. Voucher specimens have been deposited at the herbarium of the Nutraceuticals, Laila Impex, R&D centre, Vijayawada, AP, India. The collected plant material was authenticated by Dr. K. N. Reddy, Scientist, Dept. of Taxonomy, Laila impex, R&D centre. The plant was dried under shade at Dept. of Taxonomy, Laila impex, R&D centre.

Extraction, Fractionation and Isolation

Different parts of *Ipomoea staphylina* plant (500g each) were refluxed with methanol and hydro alcohol (60%methanol in water); and different extracts so obtained were named as ISWM (whole plant methanolic extract), ISWH (whole plant hydroalcoholic extract), ISSM (stem methanolic extract), ISSH (stem hydro alcoholic extract), ISRM (root methanolic extract), ISRH (root hydro alcohol extract), ISLM (leaves methanolic extract), ISLH (leaves hydro alcoholic extract), ISFM(flower methanolic extract), ISFH(flower hydro alcoholic extract). Then all the extracts were subjected to *in vitro* antioxidant, antidiabetic and anti-inflammatory pharmacological activities. Based on the results of invitro activities leaves of the plant *Ipomoea staphylina* is selected for isolation of compounds by column chromatography.

The dried leaves of *Ipomoea staphylina* (500 g) were refluxed in methanol (MEOH) for 4 h (4 lit$\times$4). The total filtrate was then concentrated to dryness *in vacuo* at 40 ^{o}C to render the MEOH extract (40 g). A portion of methanolic extract (35 g) was first chromatographed over a Silica gel column, using 80% ethyl acetate in hexane, ethyl acetate, 1% methanol in ethyl acetate, 5% methanol in ethyl acetate, 8% methanol in ethyl acetate and 10% methanol in ethyl acetate to afford eleven fractions (F01-F11). The ethyl acetate fraction (F3) gave Sitosteryl-3-O-β-D-glucoside **1** (30 mg). The 8% methanol in ethyl acetate fraction (F8) gave deoxy chiro inositol **2** (20 mg). The isolated compounds were identified and characterized by spectroscopic methods including IR; NMR and MASS; Compounds Sitosteryl-3-O-β-D-glucoside[8] (**1**) and Deoxy chiro inositol [9-10] (**2**) were identified by comparing the data with published data (Fig. 1).

In vitro studies

Anti-oxidant Activity

DPPH Radical Scavenging Activity

DPPH radical scavenging activities of all the fractions were determined by the method of Blois[16]. Initially, 0.2 mL of the fractions at a concentration of 25, 50, 75 and 100 μg/mL was mixed with 1mL of 0.2 mM DPPH (dissolved in methanol). The reaction mixture was incubated for 20 minutes at 28 °C under dark condition. The control contained all reagents except the extract fraction while methanol was used as blank. The DPPH radical scavenging activity was determined by measuring the absorbance at 517 nm using a spectrophotometer. The DPPH radical scavenging activity (%) of the sample was calculated as

$$\%\ \text{DPPH scavenging activty} = 1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

Superoxide Anion Radical Scavenging Activity

Superoxide radical scavenging activity of leaf methanolic extract of *Ipomoea staphylina* was determined by Nitro Blue Tetrazolium (NBT) riboflavin photo reduction method of Mccord and Fridovich[17]. The assay mixture contained EDTA solution (6.6 mM) containing NaCN (3 µg), riboflavin (2 µM), NBT (50 µM), test substances and phosphate buffer (67 mM, pH 7.8) in a final volume of 3 ml. The absorbance at 560 nm were measured before and 15 min after illumination (Table 1). All tests were run in triplicate and mean values were used to calculate percentage scavenging ability and IC_{50} values were calculated using linear regression analysis.

The superoxide anion radical scavenging activity (%) was calculated as

$$\%\ \text{superoxide scavenging activty} = 1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

Table 1:IC_{50} values of methanolic and hydro alcoholic extracts of *Ipomoea staphylina* stem (ISSM, ISSH), flower (ISFM, ISFH), leaves (ISLM, ISLH), root (ISRM, ISRH), whole plant (ISWM, ISWH) for various *in-vitro* antioxidant, antidiabetic and anti-inflammatory activities.

S.No	Sample	DPPH IC50 (µg/ml)	Superoxide IC50 (µg/ml)	α-glucosidase IC50 (µg/ml)	α- amylase IC50 (µg/ml)	5-Lox IC50 (µg/ml)
1	ISSH	17.67	15.54	46.76	>200	>25
2	ISSM	37.87	29.06	74.78	>200	>25
3	ISFM	63.85	89.64	73.46	148.7	>10
4	ISFH	81.03	31.17	>150	>200	>20
5	ISLM	32.77	40.99	66.91	>200	>25
6	ISLH	38.23	16.02	88.05	>200	>10
7	ISRM	81.92	93.34	90.08	>200	>10
8	ISRH	71.33	38.01	53.26	>200	>20
9	ISWM	37.71	98.14	97.00	>200	>25
10	ISWH	52.82	29.80	91.50	>200	>10
11	Std	3.41	0.62	0.009	33.59	3.97

Anti-diabetic Activity

α-Glucosidase inhibitory activity

The enzyme α- glucosidase inhibitory activity is determined by incubating solution (0.1 mL) of an enzyme preparation with 0.2 M Tris buffer, pH 8.0 (1.0 mL) containing various concentrations of extract at 37 ºC for 60 minutes by using glucose as working standard. The reaction mixture is heated for two minutes in boiling water bath to stop the reaction. The amount of liberated glucose is measured by glucose oxidation method[18].

(Assay condition 37 ºC ± 0.1 ºC, pH 8.0, O.D at 540 nm). The percentage inhibition is calculated as

$$\%\ \text{inhibition} = \frac{\text{Enzyme activity of the control} - \text{Enzyme activity of the extract}}{\text{Enzyme activity of the control}} \times 100$$

α-Amylase inhibitory activity

A starch solution (0.1% w/v) was obtained by stirring 0.1 g of potato starch in 100 mL of 16 mM of sodium acetate buffer. The enzyme solution was prepared by mixing 27.5 mg of α-amylase in 100 ml of distilled water. The colorimetric reagent is prepared by mixing sodium potassium tartarate solution and 3, 5 di nitro salicylic acid solution 96 mM. Both control (Acarbose) and plant extracts were added with starch solution and left to react with α-amylase solution under alkaline conditions at 25 ºC. The reaction was measured over 3 minutes. The generation of maltose was quantified by the reduction of 3, 5 dinitro salicylic acid to 3-amino-5- nitro salicylic acid. This reaction is detectable at 540 nm[19]. (Temperature 25 ºC ± 0.1 ºC, pH 4.8; O.D. at 540 nm).

$$\%\ \text{inhibition} = \frac{\text{Enzyme activity of the control} - \text{Enzyme activity of the extract}}{\text{Enzyme activity of the control}} \times 100$$

Anti-inflammatory Activity

In vitro 5-Lipoxygenase inhibition

5-LOX enzyme inhibitory activity of *Ipomoea staphylina* extracts was measured using the method of Reddanna *et al.*[20] modified by Ulusu *et al.*[21]. The assay mixture contained 80 mM linoleic acid and 10 µl potato 5-LOX in 50 mM phosphate buffer (pH 6.3). The reaction was initiated by the addition of enzyme buffer mix to linoleic acid and the enzyme activity was monitored as the increase in absorbance at 234 nm. The reaction was monitored for 120 sec and the inhibitory potential of the test substances was measured by incubating various concentrations of test substances for two minutes before addition of linoleic acid. All assays were performed in triplicate. Percentage inhibition was calculated by comparing slope of test substances with that of enzyme activity.

RESULTS AND DISCUSSION

In the DPPH radical scavenging assay, antioxidants react with the DPPH radicals, which is stable free radical and exists naturally in deep violet color, to turn into a yellow colored α,α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the radical scavenging potential of the antioxidant. DPPH radical scavenging activity of stem hydro alcoholic and leaf methanolic extract of *Ipomoea staphylina* is found to be potent compared to other extracts and results are compared with standard as having IC_{50} values 17.67 µg/ml, 32.77 µg/ml respectively (Fig.2). It has been found that phenolics, flavonoids and tocopherols reduce the DPPH radicals by their hydrogen donating ability.

In this study, the superoxide anion scavenging effects of various extracts were analyzed and the results are given in Fig. 3. Among the extracts tested, stem hydro alcoholic and leaf hydro alcoholic extract of *Ipomoea staphylina* is found to be potent compared to other extracts having IC_{50} values 15.54 µg/ml, 16.02 µg/ml respectively. The superoxide anion scavenging activity might be due to the action of polyphenolic compounds.

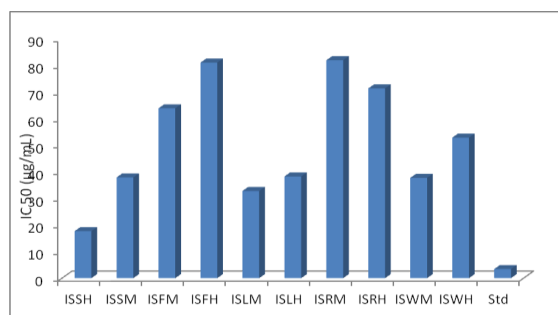

Fig. 2: DPPH radical scavenging activity of methanolic and hydro alcoholic extract of *Ipomoea staphylina* stem (ISSM, ISSH), leaves (ISLM, ISLH), flower (ISFM, ISFH), root (ISRM, ISRH), whole plant (ISWM, ISWH).

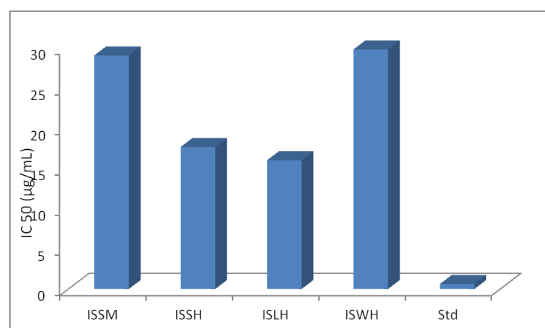

Fig 3:Superoxide radical scavenging activity of methanolic extract of *Ipomoea staphylina* stem (ISSM) and hydroalcoholic extract of *Ipomoea staphylina* stem (ISSH), leaves (ISLH), whole plant (ISWH).

Also the *in-vitro* α-glucosidase inhibitory activity, *in-vitro* α-amylase inhibitory activity and 5-lox enzyme inhibitory activity of various extracts were analyzed. The 50 % inhibitory concentration of various extracts of *ipomoea staphylina* was found to be very less potent compared to standard. So extracts of *Ipomoea staphylina* are devoid of *in-vitro* antidiabetic and anti-inflammatory activity.

Methanolic extract of *Ipomoea staphylina* leaves was selected for further studies, because methanolic extract was found to be potent for *in-vitro* antioxidant activity in DPPH and superoxide radical scavenging activity. It was fractionated by column chromatography. Some of the fractions were further subjected to fractionation. Some of the pure compounds were isolated from leaves methanolic extract. The fractions which were obtained in negligible amounts were excluded from fractionation.

Spectral Analysis of Isolated Compounds

Compound (1): White crystals; IR (KBr), V_{max} 3460, 2920, 2849, 1458, 1366, 1021,1655, 800 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$ + 2 drops MeoD) δ_H: 5.362 (1H, d, J = 4.8 Hz), 0.695 (3H, s), 0.909 (3H, s), 0.83 (3H, J = 7.2 Hz), 0.80 (3H, J = 7.2 Hz), 0.77 (3H, J = 7.22 Hz), 4.409 (1H, t, J = 7.76 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$ + 2 drops MeoD) δ_c: 37.43 (C-1), 29.81 (C-2), 79.38 (C-3), 38.91 (C-4), 140.48 (C-5), 122.30 (C-6), 32.08 (C-7), 32.08 (C-8), 50.40 (C-9), 36.89 (C-10), 21.11 (C-11), 38.74 (C-12), 42.51(C-13), 56.95 (C-14), 24.43 (C-15), 28.36 (C-16), 56.05 (C-17), 11.96 (C-18), 19.42 (C-19), 36.29 (C-20), 18.89 (C-21), 39.55 (C-22), 26.06 (C-23), 46.09 (C-24), 29.15 (C-25), 19.87 (C-26), 19.08 (C-27), 23.07 (C-28), 12.6 (C-29), 101.30 (C-1), 73.76 (C-2), 76.59 (C-3), 70.48 (C-4), 75.88 (C-5), 62.20 (C-6). LC-MS (positive) *m/z*: 575.5 $[M-H]^-$.

Compound (2): White solid; IR (KBr), V_{max} 3311, 1422.1, 1278, 1069, 1049 cm^{-1}; ^{1}H-NMR (400 MHz, D_2O) δ_H: 1.941 (1H, d, t, J = 14, 4.4Hz), 1.764 (1H, ddd, J = 14.4, 11, 3.2Hz), 3.664 (1H, dd, J = 9.6, 3.2Hz), 3.514 (1H, t, J = 9.6Hz), 3.717 (1H, ddd, J = 9.2, 4.4, 20Hz), 3.883 (1H, t, J = 3.6Hz), 3.974 (1H, q, J = 3.2Hz); ^{13}C-NMR (100 MHz, D_2O) δ_c: 33.36 (C-1), 71.14 (C-2), 74.76 (C-3), 69.06 (C-4), 72.39 (C-5), 68.70 (C-6). LC-MS (positive) *m/z*: 163 $[M-H]^-$.

On comparing data generated with that of published literature, it was confirmed that compounds isolated from methanolic extract of leaves of *Ipomoea staphylina* were Sitosteryl-3-O-β-D-glucoside[13] (1) and Deoxy chiro inositol [14-15] (2).

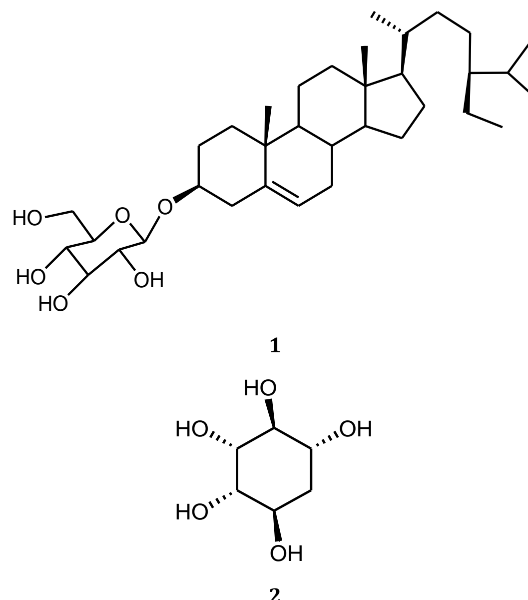

Fig1: Compounds isolated from leaves of *Ipomoea staphylina*

Ipomoea staphylina leaves methanolic extract was having DPPH radical scavenging activity. Sitosteryl-3-O-β-D-glucoside isolated was not tested for their DPPH radical scavenging activity, as it was soluble only in chloroform. Deoxy chiro inositol was not having antioxidant activity (DPPH radical scavenging activity).

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