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Research Article

Potential Investigation of Antioxidant and Anti-inflammatory Activities in *in-vitro* Root tubers of *Holostemma annulare* (Roxb.) K Schum

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ABSTRACT

Holostemma annulare is a twining perennial shrub belonging to the family *Asclepiadaceae* with therapeutic and pharmacological potentialities. The root tuber of the plant is rich in antioxidants and has been suggested as potential source of anti-inflammatory compounds. Thus, this study evaluated the antioxidant and anti-inflammatory activities of the methanolic *in-vivo* and *in-vitro* root tuber extracts of the *H. annulare* with widespread use in folk medicine. Antioxidant activity was evaluated by DPPH, nitric oxide, hydrogen peroxide, superoxide scavenging and reducing power activity assays. In addition, *in-vitro* anti-inflammatory activity of the methanol root tuber extracts was evaluated by proteinase inhibition assay, cyclooxygenase (COX) and lipoxygenase (LOX) assays. The results revealed that *in-vitro* root tuber extracts possess significant antioxidant and anti-inflammatory activities to the extent of *in-vivo* tubers and thus can be explored as an important source of novel antioxidant and anti-inflammatory agents. This application also emphasizes the potential utilization of *in-vitro* tubers of the species for drug formulations.

Introduction

Plants have been used as medicines for treating various ailments in different cultures and religions for thousands of years.[1] Medicinal plants heal and cure many diseases because they contain some organic compounds that produce definite physiological action on the human body. These bioactive substances are mainly divided into primary and secondary metabolites based on their functions in plant metabolism. The exploitation of plants and plant products for the pharmaceutical purpose has been increasing tremendously in recent years. These natural drugs are gaining popularity because of fewer side effects, patient betterment, relatively less expensive and recognition due to a long historical background of usage.[2] Different metabolic activities of the body and the influence of environmental factors like pollutants, radiation, toxins and chemicals can generate excess free radicals in the cells. This creates an imbalance between the production of reactive oxygen species (ROS) and defensive antioxidant molecules in the cells, which leads to oxidative stress in the physiological system, thereby resulting in damage of DNA, lipids and proteins, causing various diseases such as arthritis, cancer, inflammation, infertility and aging. Antioxidant molecules can stabilize or deactivate these free radicals before they attack cells and hence are absolutely critical for maintaining optimal cellular and systemic health and well-being.[3]

Inflammation, either acute or chronic, is usually a body response to tissue damage and to a number of systemic malfunctions, including asthma, atherosclerosis, arthritis, physical injury and infection, amongst many others.[4] Acute inflammation may be an initial response of the

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body to harmful stimuli, while in chronic inflammation, the inflammatory response is out of proportion resulting in damage to the body. Regarding the mechanism of inflammation, the metabolism of arachidonic acid has a key role.[5] Two cyclooxygenases (constitutive COX-1 and inducible COX-2) and lipoxygenase (5-LOX) enzymes are responsible for the transformation of arachidonic acid into the potent biologically active lipid mediators that are intimately involved in inflammation.[6] Steroidal and non-steroidal drugs like aspirin, ibuprofen, diclofenac, ketoprofen, and naproxen are current medicaments in treating acute inflammatory diseases and these drugs block COX-1 and COX-2 enzymes. However, long-term usage of these drugs results in adverse side effects and damages human biological systems causing renal failure and gastrointestinal damage.

The medicinal plant *Holostemma annulare* (Roxb.) K. Schum., popularly known as Jivanti, a perennial lactiferous climber belonging to Asclepiadaceae family growing in the tropical Western Ghats, has traditionally been used in indigenous system of medicine for maintaining youthful vigor and potentiality. The root tubers of this species are medicinally important and are useful in ophthalmopathy, orchitis, cough, fever, burning sensation, stomachalgia and also as an expectorant, tonic, stimulant and galactagogue.[7] The major ingredient of the drug "Jivanti" is the tuberous roots of *H. annulare* , which is one of the main herbs in the medicine system. The root tubers possess terpenoid sugars which are responsible for their medicinal properties. They are a rich source of vitamin A, and a mash of the same in cold milk is a remedy for diabetes. For the preparations of ayurvedic drugs in south indian pharmacies, at least 150 tonnes of root tubers are required per annum. This led to the scarcity of plants and the global demand for *H. annulare* herbal materials has drastically increased recently. Biotechnological interventions could only be an alternative for producing large quantities of harvestable parts within a short time. To meet the same, we have induced *in-vitro* root tubers in *H. annulare* . The present investigation aimed to evaluate different phytochemicals and screening some bioactive potentialities, especially the antioxidant and anti-inflammatory activity of the *in-vivo* and *in-vitro* root tubers of the species. Perusal of the literature reveals that there are no reports in both aspect, including *in-vitro* root tuber induction in these taxa.

Materials And Methods

Collection of Plant Material

H. annulare in-vivo root tubers were collected from the homestead cultivation at Vembayam, Thiruvananthapuram District, Kerala, India and the *in-vitro* root tubers were produced from *in-vitro* culture.

Preparation of Plant Extract

The collected root tubers were cut into small pieces and washed thoroughly under running tap water, subsequently with distilled water, then allowed to dry in the shade and powdered. The powdered material was subjected to soxhlet extraction using methanol as the solvent for 8 hours at the boiling point of the solvent. The extract was concentrated to dryness and the residue was transferred to a sample bottle and stored for further studies.

Preliminary Phytochemical Investigation

Qualitative Analysis

The methanol extract of *in-vivo* and *in-vitro* samples were subjected to qualitative phytochemical screening to identify the presence or absence of various bioactive compounds such as alkaloids (dragendroff's test and wagner's test), flavanoids (ammonium test, alkaline reagent test, shinoda test), phytosterols/terpenoids (liebermann-burchard's test), tannin and phenol (ferric chloride test), triterpenoids (salkowski test), anthraquinone glycosides, carbohydrates (molisch's test, fehling's test), proteins (biuret test) and saponins (foam test) according to the standardized procedures described by Harbone[8] and Sofowora.[9]

Quantitative Analysis

Quantitative analysis were carried out in the methanolic root tuber extract of (*in-vivo* and *in-vitro)* of *H. annulare* samples to estimate the phytoconstituents *viz*. proteins,[10] carbohydrates,[11] reducing sugar,[12] tannins,[13] alkaloids,[8] phenols,[14] flavonoids,[15] and triterpenoids[16] were using standard procedures.

Determination of *In-vitro* Antioxidant Activities

The free radical scavenging activity of the methanol extracts of two selected samples of *H. annulare* root tubers (*in-vivo* and *in-vitro*) was determined by using various *in-vitro* assays such as DPPH (2, 2-Diphenyl -1-picryl hydrazyl),[17] nitric oxide,[18] hydrogen peroxide,[19] superoxide[20] radical scavenging assays and reducing power activity[21] with standard drugs. The antioxidant activity of the sample was calculated and expressed as IC_{50} (the 50% concentration of extracts that inhibits the formation of free radicals).

DPPH Assay

The antioxidant activities of methanolic root tuber extracts were evaluated based on the radical scavenging effect using a stable DPPH assay.[17] The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm. Ascorbic acid (10 mg/mL DMSO) was used as a reference. Different concentrations of a sample such as 12.5 μgmL^{-1} to 200 $\mu g\ mL^{-1}$ from stock solution of 10 $\mu g\ mL^{-1}$ were made up to a final volume of 20 µL with DMSO and 1.48 mL DPPH

(0.1 mM) solution was added. Control without the test compound, but an equivalent amount of distilled water was taken. The reaction mixture was incubated in dark condition at room temperature for 20 minutes. Thereafter, the absorbance was read at 517 nm and 3 mL of DPPH was taken as control. DPPH scavenging in percentage was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

Nitric Oxide Scavenging Assay

Different concentrations of a sample such as 125–2000 $mgmL^{-1}$ from a stock concentration of 10 $mgmL^{-1}$ were mixed with sodium nitroprusside in phosphate-buffered saline pH 7.4 and incubated at 25°C for 30 minutes. Control without the test compound, but an equivalent amount of distilled water was taken. After 30 minutes, 1.5 mL of the incubated solution was removed and diluted with 1.5 mL of griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% N-(1-naphthyl)ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-(1 naphthyl)ethylene diamine dihydrochloride was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard gallic acid as follows:

$$\% \text{ inhibition} = \frac{\text{Control- Test}}{\text{Control}} \times 100$$

Superoxide Radical Scavenging Assay

Superoxide is biologically important as it can form singlet oxygen and hydroxyl radicals and the over production of these radicals contributes to redox imbalance and associated with harmful physiological consequences. Different concentrations of sample such as 125–2000 μgmL^{-1} from a stock solution of 10 $mgmL^{-1}$, 0.05 mL of riboflavin solution (0.12 mM), 0.2 mL of Ethylenediaminetetraacetic acid (EDTA) solution (0.1 M) and 0.1 mL NBT (nitro-blue tetrazolium) solution (1.5 mM) were mixed in test tube and the reaction mixture was diluted up to 2.64 mL with 0.067 M phosphate buffer. A control was taken without the test compound, but an equivalent amount of distilled water. The absorbance of the solution was measured at 560 nm after illumination for 5 minutes of incubation in fluorescent light and also measured after illumination for 30 minutes. at 560 nm on UV-visible spectrophotometer. OD was calculated. The percentage of inhibition was determined as follows:

$$\% \text{ inhibition} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

Hydrogen Peroxide Scavenging Assay

A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Different concentration of sample such as 125–2000 $\mu g.mL^{-1}$ from a stock concentration of 10 $mg.mL^{-1}$ was added to H_2O_2 solution (0.6 mL). A control was taken without the test compound, but an equivalent amount of distilled water. OD was read at 230 nm after 10 minutes of incubation. Ascorbic acid (10 $mg.mL^{-1}$) was used as standard. Percentage inhibition was calculated as,

$$\% \text{ inhibition} = \frac{\text{Control- Test}}{\text{Control}} \times 100$$

Reducing Power Activity

Different concentrations of sample such as 125–2000 $mgmL^{-1}$ from a stock concentration of 10 $mg.mL^{-1}$ were mixed with 2.5 mL of phosphate buffer (200 mM) (pH 6.6) to which 2.5 mL of 1% potassium ferric cyanide was added and boiled for 20 minutes at 50°C. A control was taken without the test compound, but an equivalent amount of distilled water. After incubation, 2.5 mL of 10% TCA was added to the mixtures followed by centrifugation at 5000 rpm for 10 minutes. The upper layer (5 mL) was mixed with 5 mL of distilled water and 1-mL of 0.1% ferric chloride was added to it and the absorbance was read at 700 nm. Quercetin in 10 $mg.mL^{-1}$ DMSO was used as a reference.

Determination of *In-vitro* Anti-inflammatory Activity

In-vitro anti-inflammatory activity of the methanol extracts of two selected samples (*in-vivo* and *in-vitro* root tubers) *of H. annulare* were evaluated by analyzing the proteinase inhibitory activity, COX and LOX activity.

Proteinase Inhibitory Activity

Proteinase inhibitory activity of the *in-vivo* and *in-vitro* root tuber extracts were determined by the method of Sakat *et al.*[22]. Different concentrations of the sample such as 62.5–500 $\mu g/mL^{-1}$ from a stock concentration of 10 $\mu g/mL^{-1}$ were used for the study. The reaction mixture (2 mL) was containing 0.06 mg trypsin, 1-mL, 20 Mm tris HCl buffer (pH 7.4) and 1-mL test sample at different concentrations. The mixture was incubated at 37°C for 5 minutes. Then 1-mL of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 minutes. Then 2 mL of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged at 3000 rpm for 10 minutes. The absorbance was measured using UV-visible spectrophotometer at 200 nm (SL119, Systronics) against the buffer as blank. The percentage of proteinase inhibitory activity was calculated using the following formula:

$$\% \text{ inhibition} = 100 - \frac{(OD_{\text{Test solution}} - OD_{\text{product}})}{(OD_{\text{Test control}})} \times 100$$

Cycloxygenase (COX) and Lipoxygenase (LOX) Assay

Cell lines

RAW 264.7 cells were grown to 60% confluence followed by activation with 1-µL lipopolysaccharide (LPS) (1 $\mu g\ mL^{-1}$).

LPS stimulated RAW cells were exposed with different concentration of sample solution. Diclofenac sodium, a standard anti-inflammatory drug in varying concentration corresponding to the sample were also added and incubated for 24 hours. After incubation the anti-inflammatory assays were performed using the cell lysate.

COX Assay

The COX activity was performed according to the modified method of Walker and Gierse.[23] The cell lysate in tris-HCl buffer (pH 8) was incubated with glutathione 5 mML^{-1} and hemoglobin 20 μgL^{-1} for 1-minute at 25°C. The reaction was initiated by adding arachidonic acid 200 mML^{-1} and terminated after 20 minutes of incubation at 37°C by adding 10% trichloro acetic acid in 1-N hydrochloric acid. After the centrifugal separation and the addition of 1% thiobarbiturate, COX activity was determined by reading absorbance at 632 nm. Percentage inhibition of the enzyme was calculated as,

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

LOX Assay

The determination of 5-LOX activity was assayed by the method of Axelrod et al.[24] Briefly, the reaction mixture (2 mL final volume) contained tris-HCl buffer (pH 7.4), 50 µL of cell lysate and sodium linoleate (200 µL; 10 $mgmL^{-1}$). The LOX activity was monitored as the difference in absorbance at 234 nm, which reflects the formation of 5-hydroxyeicosatetraenoic acid from linoleate. Percentage inhibition of the enzyme will be calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Statistical Analysis

All the experiments were conducted in six replicates repeated thrice. Mean values were compared by t-test at $p \leq 0.01$.

Results

Phytochemical Investigation

The result of phytochemical analysis revealed the presence of a variety of phyto-constituents in both samples *(in-vivo* and *in-vitro* root tuber extracts) of *H. annulare* . The *in-vivo* sample showed the presence of most of the phytochemicals viz, alkaloids, flavanoids, triterpenes, tannins, phenols, proteins, phytosterols with exception of saponins and anthroquinones (Table 1). The quantity of different phytoconstituents like alkaloids, phenols, and flavanoids were significantly higher in the *in-vivo* root tuber samples compared to the methanol extract of the *in-vitro* root tuber. Quantitative analysis of different phytoconstituents showed the highest value for soluble sugar content (12.68 mgg^{-1}). The amount of proteins and reducing sugar were 0.98 mgg^{-1} and 0.08 mgg^{-1}), respectively in the methanol extract of *in-vivo* root tuber of *H. annulare* . The results suggested that methanol root extracts of *in-vivo*

Table 1: Phytochemical investigation of methanolic root tuber extracts of *H. annulare*

Phytochemical	*Test performed*	*Inference of phyto-constituents*	*In-vivo root tuber*	*In-vitro root tuber*
Alkaloids	Wagner's test	Formation of yellow or brown precipitate	+++	++
	Dragendroff's test	Formation of a reddish brown precipitate	++	++
Flavanoids	Alkaline test	A yellow colour observed at ammonia layer	+	++
	Shinoda test	A pink, scarlet, crimson red or occasionally green to blue colour appeared after few minutes	+++	+
Triterpenoids	Salkowski test	A red brown color formed at the interface	++	+
Tannins	$FeCl_3$ test	Formation of bluish black colour	+	+
Phenol	-	Formation of greenish black colour	+	+
Antraquinones	-	Appearance of Red colour	-	-
Carbohydrates	Molisch's test	A reddish violet or purple ring at the junction of two liquids	++	+
	Fehling's test	Brick red precipitate	-	-
Saponins	Foam test	Stable foam	-	-
Protein	Biurette test	Formation of purple or violet color	+	+
Phytosterol	Libermann-burchards	A brown ring formation at the junction	++	-

(+ Present, - Absent)

Table 2: Quantitative analysis of phytochemicals in methanol root tuber extracts of *H. annulare*

Name of the compound	*Quantity (mgg^{-1})*	
	In-vivo root tuber	*In-vitro root tuber*
Carbohydrate	12.68 ± 0.010^a	11.23 ± 0.005^b
Reducing sugar	0.80 ± 0.010^a	0.76 ± 0.005^b
Proteins	0.98 ± 0.002^a	0.94 ± 0.001^a
Alkaloids	7.62 ± 0.010^a	7.01 ± 0.015^a
Phenols	10.47 ± 0.005^a	10.29 ± 0.020^a
Flavanoids	1.88 ± 0.010^a	1.65 ± 0.030^b
Tannin	0.42 ± 0.050^b	0.74 ± 0.015^a
Triterpenoid	0.31 ± 0.009^a	0.29 ± 0.020^b

and *in-vitro* root tuber contained 1.88 and 1.65 mgg^{-1} of flavanoids, 7.62 and 7.01 mgg^{1} of alkaloids, respectively. Quantity of tannin estimated as tannic acid equivalent (TE mgg^{-1}) in the *in-vivo* and *in-vitro* root tubers were found ranging from 0.42 mgg^{-1} and 0.74 mgg^{-1}. In the present study the phenolic content in methanol extract of *in-vivo* sample was slightly high (10.47 mgg^{-1}) than *in-vitro* sample (10.29 mgg^{-1}). In comparison to alkaloids, flavonoids, phenolics and tannin, the total triterpenoid content detected was substantially low in methanol extract of *in-vitro* sample (0.29 mgg^{-1}) than the *in-vivo* sample and was found to be in the order *in-vivo* triterpenoid (0.312 mgg^{-1}) > *in-vitro* triterpenoid (0.285 mgg^{-1}) (Table 2). The amount of phyto-constituents varied in little extent in both the samples and the variation was statistically significant only at 1% level of significance and the variation is correlated to some other factors viz. the maturity of the samples and so on. These results indicated that the *in-vitro* root tuber sample can also be utilized as a better source of phytocomponents for drug preparations than in-vivo plant.

Data represents mean values of six replicates repeated thrice. Mean values followed by the same letter in the superscript in a row do not differ significantly based on t-test at $p \leq 0.01$.

In-vitro Antioxidant Activities

The antioxidant activities were analysed with the two samples using the following assays.

DPPH Scavenging Assay

The DPPH free radical scavenging activity of the methanol *in-vivo* root tuber extract of *H. annulare* showed slightly highest scavenging activity (%inhibition of 15.18, 25.75, 33.73, 51.13 and 58.78 at 12.5, 25, 50, 100, 200 µgmL^{-1} concentrations, respectively), while the methanolic *in-vitro* root tuber extract showed DPPH radical scavenging ability with % inhibition of 13.21, 22.16, 30.27, 40.35, and 53.54 at 12.5, 25, 50, 100, 200 µgmL^{-1} concentrations, respectively (Fig. 1). The methanol extracts of the two samples exhibited a dose dependent increase in DPPH scavenging activity. The results of the free radical scavenging activity of *H. annulare* assessed by DPPH test and amount of the sample needed for 50% inhibition of free radical activity, i.e., IC_{50} values are summarized below Fig. 1. Lower IC_{50} value suggests higher antioxidant activity. Based on the results found the antioxidant activity of *H. annulare in-vivo* root tuber methanol extract (IC_{50}: 85.953) µg mL^{-1} is comparable with the standard antioxidant of ascorbic acid.

Nitric Oxide Scavenging Assay

Nitric oxide plays a significant role in a variety of inflammatory processes and it is an unstable free radical involved in many biological process which are associated with several diseases .The scavenging activity of nitric oxide by the *in-vivo* and *in-vitro* root tubers extracts has increased in a dose dependent manner. The *in-vivo* extracts showed maximum activity of 67.21% at 2000 µgmL^{-1} where as that of gallic acid was 92.80% at the same concentration (Fig. 2). The IC_{50} values were found to be 684.062 µgmL^{-1} for *in-vivo* samples, 766.128 for *in-vitro* root tuber and 108.809 µgmL^{-1} for gallic acid. The nitric oxide radical scavenging activity of *H.annulare in-vivo and in-vitro* root tubers extracts increased up to 50% at the concentrations of 1000 and 2000 µgmL^{-1}.

H_2O_2 Scavenging Assay

The hydrogen peroxide scavenging activity of *in-vivo* and *in-vitro* root tuber extract of *H. annulare* was evaluated and compared with ascorbic acid and the results are given in Fig. 3. The methanolic *in-vivo* root tuber extract showed

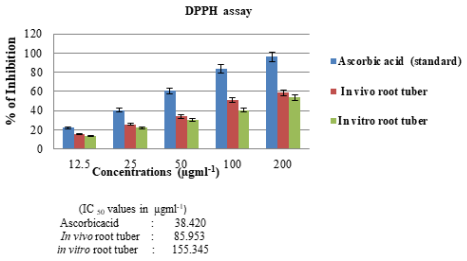

Fig. 1: DPPH scavenging assay of *H. annulare* methanol root tuber extracts

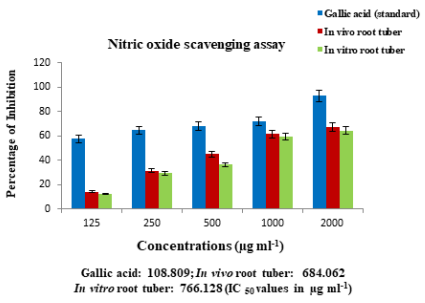

Fig. 2: Nitric oxide scavenging assay of *H. annulare* methanol root tuber extracts

significant scavenging activity of H_2O_2 in a concentration dependent manner with an IC_{50} values of 711.585 μgmL^{-1} with percentage of inhibition (61.56 %) whereas the IC_{50} values for *in-vitro* extracts and standard ascorbic acid was 1391.649 μgmL^{-1} and 223.499 μgmL^{-1}, respectively.

Superoxide Free Radical Scavenging Assay

The superoxide scavenging assay revealed that the methanol extracts of root tuber (*in-vivo*) of *H. annulare* showed the highest percentage (71.74%) of inhibition at 2000 μgmL^{-1} concentration with IC_{50} value of 1237.969 μgmL^{-1}, while *in-vitro* root samples showed almost similar activity with IC_{50} value of 1342.894 μgmL^{-1}, and the IC_{50} value of standard was 238.357 μgmL^{-1}. The results are shown in the Fig. 4.

Reducing Power Activity

Fig. 5 shows the reducing ability of methanol extract of *in-vivo* and *in-vitro* samples of *H. annulare* compared to ascorbic acid. Absorbance of the solution increased when the concentrations increased and a highest absorbance indicated a higher reducing power. Among this two extracts tested, *in-vitro* root tuber extract exhibited higher reducing activity and the reducing power increased with concentration of each extract.

Anti-inflammatory Activities

The anti-inflammatory activity was analysed with the *in-vitro* and *in-vivo* root samples using the following assays and the findings emphasized that the potentiality of *in-vitro* root samples were on par with that of *in-vivo* roots.

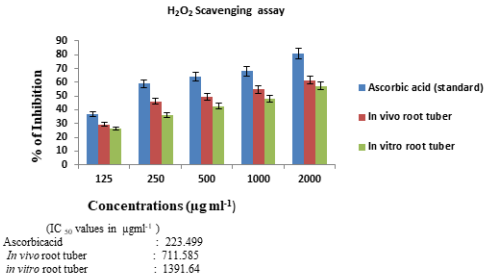

Fig. 3: H_2O_2 Scavenging assay of *H. annulare* methanol root tuber extracts

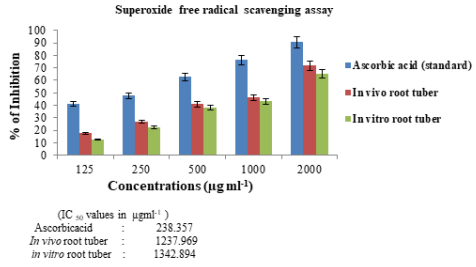

Fig. 4: Superoxide free radical scavenging assay of *H. annulare* methanol root tuber extracts

Proteinase Inhibitory Activity

The proteinase inhibitory action of two root extracts of *H. annulare* showed significant action and the results are shown in Fig. 6. Diclofenac sodium was used as the standard and the OD of test control was 0.9113. At various concentrations *viz.* 62.5, 125, 250 and 500 μgmL^{-1}, the percentage of inhibition by standard was found to be 74.67, 81.25, 85.94 and 90.64%. The inhibition levels were observed within the range of 11.22–62.53% in two samples. The *in-vitro* root tuber samples showed significantly higher proteinase inhibition at 500 μgmL^{-1} with 62.53% inhibition and the IC_{50} = 360.696 μgmL^{-1} while the *in-vivo* samples exhibited 52.84% inhibition with IC_{50} = 388.926 μgmL^{-1}.

COX Assay

In-vivo and *in-vitro* methanol root tuber extracts of the *H. annulare* were analysed for anti- inflammatory activity by COX assay and compared with the standard drug diclofenac sodium. Both *in-vivo* and *in-vitro* extracts showed strong inhibitory activity of the COX enzyme with IC_{50} values 67.92 and 72.01mgmL^{-1}, respectively. At different concentrations *viz.* 6.25, 12.5, 25, 50, 100 μgmL^{-1}, the percentage of inhibition by standard diclofenac sodium was found to be 10.34, 26.95, 36.46, 52.86, and 60.76% for COX enzyme with an IC_{50} value of 40.78 μgmL^{-1}. The percentage COX inhibition exhibited by different concentrations (6.25, 12.5, 25, 50, 100 μgmL^{-1}) of *in-vivo* and *in-vitro* extracts were found to be 10.25, 20.73,2 8.77, 48.65 and 59.24% (IC_{50}= 67.92 mgmL^{-1}) and 10.22, 18.24, 26.44, 45.24 and 56.32% (IC_{50}= 72.01 mgmL^{-1}), respectively (Fig. 7).

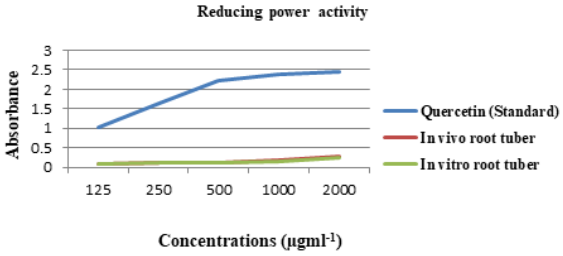

Fig. 5: Reducing power activity of *H. annulare* methanol root tuber extracts

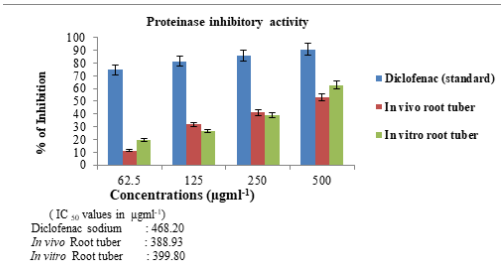

Fig. 6: Proteinase inhibitory activity of *H. annulare* methanol root tuber extracts.

LOX Assay

The anti-inflammatory activity of *H. annulare in-vivo* and *in-vitro* root tuber extracts were estimated by inhibition of lipoxgenase activity. In the present study *in-vitro* extract showed good 5-lipxoygenase inhibitory activity when compared to standard. The percentage of inhibition at various concentrations of *in-vitro* root tuber extracts (6.25, 12.5, 25, 50, 100 μgmL^{-1}) was found to be 10.06, 13.46, 24.44, 44.65 and 58.07%, while for the standard diclofenac sodium, the percentage of inhibition at the same concentrations were 10.35, 26.96, 36.45, 52.86 and 60.76%, respectively (Fig. 8). From the observations highest inhibitory effect was observed for *in-vitro* root tuber extracts with an IC_{50} value 71.07 μgmL^{-1}. Among the two extracts *in-vitro* root tuber extract inhibited high LOX activity than *in-vivo* extract. The percentage of inhibitory activity *in-vivo* extract against COX enzyme (IC_{50} = 67.92 μgmL^{-1}) were generally higher to that of LOX (IC_{50} = 84.83 μgmL^{-1}) at the same concentration. The results indicated that inhibitory effect of each plant extract on enzyme were dose dependent. Screening of the crude extract at concentration 100 μgmL^{-1} revealed that the methanol extract had the greatest potential to inhibit both COX and LOX.

Discussion

Nature has served as opulent repository of medicinal plants for thousands of years and remarkable number of modern drugs has been isolated from natural sources, markedly from plant origin.[25] The preliminary phytochemical analysis of two extracts exhibited a wide range of phytoconstituents such as alkaloids, tannins, terpenoids, flavonoids, phytosterols and phenols. Among the phytochemicals, phenolics, flavonoids and terpenoids have expanded a particular interest because of their wide ranging antioxidant activities and anti-inflammatory activities.[26] Flavonoids are also known as a vitamin P elicit a wide range of therapeutic activities such as antihypertensive, anti-rheumatism, anti-diuretic, antioxidant, antimicrobial and anticancer properties.[27,28] Alkaloid exhibited promising antidiarrheal, anti-inflammatory, anticancer and antidiabetic activities and cure urinary disorders.[29,30] Phenols are highly effective anticoagulants, antioxidants, immune enhancers, hormone modulators and they modify the prostaglandin pathways, protect platelets from clumping and inhibits the enzymes which stimulates the inflammation.[31] Steroids reduce the cholesterol levels and helps in regulating immune system.[32] Present study confirmed that the samples analysed contain terpenoids which is a good constituent for anti-inflammatory and anti-microbial activities,[33] and also the terpenoids have been recognized for centuries as, anti-fungal, anti-viral, anti-parasitic, anti-hyperglycemic, anti-ulcer, analgestic, anti plasmodic, anti-allergenic, and anti-tumoral agents which are used for therapeutic uses for centuries.

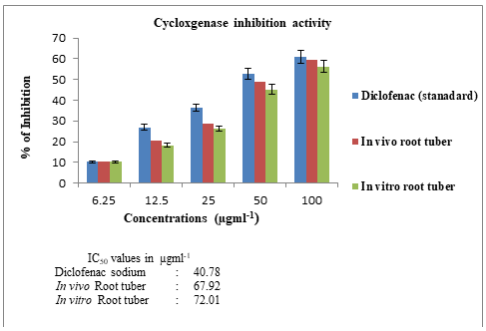

Fig. 7: Cycloxgenase inhibition activity of *H. annulare* methanol root tuber extracts.

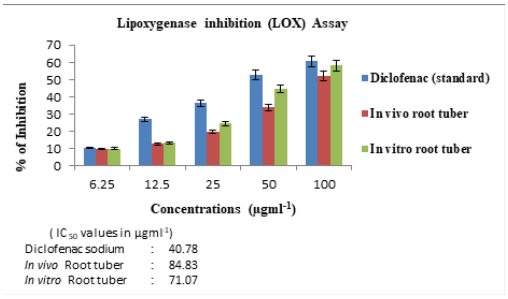

Fig. 8: Lipoxygenase inhibition (LOX) assay of *H. annulare* methanol root tuber extracts.

The ability to scavenge free radicals is generally used for the fast evaluation of the antioxidant property of samples and the present investigation has explored the same in the samples analysed here. A free radical is a molecule or atom that carries one or more unpaired electrons and is able to exist independently.[34] Free radicals are short-lived, highly reactive and unstable as they possess odd number of electrons. Subsequently, to obtain stability, free radicals can react quickly with other elements trying to catch the required electron. Meanwhile, the confronted molecule can become a free radical by losing its electron and start a chain reaction cascade causing damage to the living cell.[35] DPPH radical scavenging is regarded as a milestone for assessing the antioxidant potential of materials. The antioxidant effects on DPPH scavenging were thought to be due to capacity of their hydrogen donation capacity. DPPH is a stable free radical with maximum absorption at 517nm that can readly undergo scavenging by antioxidant. The DPPH method has based on reduction of alcoholic DPPH solutions in the presence of a hydrogen granting antioxidant. The antioxidant molecules such as ascorbic acid, tocopherol, flavanoids and tannins reduce and decolorize DPPH due to their hydrogen donating capacity.[36] Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is diffusible free radical that play many roles as an effector molecules

diverse biological system including neuronal messenger, vasodilatation, antimicrobial and anti-tumor activities.[37] NO• is usually linked with inflammation[38] and carcinogenesis.[39] Hydrogen peroxide occurs naturally at low concentration levels in the air, water, human body, plants, microorganisms and food.[40] H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (•OH) that can initiate lipid peroxidation and cause DNA damage.[41] Superoxide anion is a weak oxidant, it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress.[42] Numerous biological reactions generate superoxide anions which are highly toxic species. Hydroxyl and superoxide radicals are the free radicals that are physiologically produced by the body through its metabolic processes.[43] The immune system utilizes these reactive species in order to fight the invading pathogens so as to maintain the homeostasis inside the body. The reducing capacity of a compound may serve as an important indicator of its potential antioxidants activity.[44] The reducing activity of a compound generally depends on the presence of reductases which have been exhibited antioxidant potential by breaking the free radical chain, donating a hydrogen atom.[45]

The results explained here have revealed better scavenging ability of free radicals that has been checked with the root tuber samples. Previously, Jelly Louis[46] reported that 70% of methanol extract of root of *Holostemma adakodien* is required for 50% inhibition of super oxide free radical production, nitric oxide radical production, hydroxyl radical generation and lipid peroxide formation. Mallikarjuna *et al.*[47] confer that the methanolic root extract of *Holostemma adakodien* showed good nitric oxide scavenging activity compared to that of hexane and ethyl acetate extract. Rubesh Kumar *et al.*[48] reported that methanolic leaf extracts of *Holostemma adakodien* possess high DPPH, superoxide anion radical scavenging, nitric oxide, H_2O_2 and reducing power activity than hexane, ethyl acetate and hydroalchoholic extracts. Kaffoor *et al.*[49] reported that the antioxidant activity of aqueous and methanolic extracts of *Hemidesmus indicus* showed the good antioxidant activity. But, there are no studies regarding the comparative analysis antioxidant potentiality of *in-vivo* and *in-vitro* root samples of *H. annulare* yet.

Experiments to check the anti-inflammatory activity of the samples was also undertaken. Inflammation is the response of living tissues to injury which involves a complex array of enzyme activation, mediator release, and extravasations of fluid, cell migration, tissue breakdown and repair.[50] The enzyme COX is a membrane bound glycoprotein found in the endoplasmic reticulum of prostanoid forming cells and it has been played as a key role in inflammatory process. COX-1 and COX-2 are the two isoforms of COX enzymes, among them COX-2 involved in mediating the inflammatory process produces PGE2 from endogenous arachidonic acid. Lipoxygenases (LOXs) are a family of non-heme iron-containing dioxygenases catalyzing the biosynthesis of loukotrienes and these leukotrienes function as initiators of inflammation and their inhibition is responsible for the anti-inflammatory activity. The results of the study suggested that the methanolic root extract may reduce productions of inflammatory mediators such as; prostaglandins and leukotriene's since it significantly inhibits COX and 5-LOX, respectively. The anti-inflammatory activity of extract may be due to the presence of flavonoids and terpenoids which possess various biological properties related to antioxidant, antinociceptive, and anti-inflammatory mechanisms by targeting ROS and prostaglandins which are involved in the late phase of acute inflammation and pain perception.[51-53] The findings confer that phyto-constituents in *H. annulare* root extract were capable of inhibiting COX and LOX enzyme of the arachidonic acid cascade in human cellular system. In his studies, Jelly Lousis reported that 70% methanolic extract of *H. adakodien* root effectively inhibited the inflammation induced by formalin and carrageenan. Yasodha Krishna[54] also studied that the ethanolic extract of leaves of *H. adakodien* possess good anti-inflammatory activity against albino rats, while the ethanolic extract of latex protease of *H. adakodien* possessed good anti-inflammatory activity.[55]

Thus, the antioxidant activity of *in-vivo* and *in-vitro* root tubers of methanolic extracts showed good free radical scavenging activity because of the presence of a high amount of flavanoids and terpenoids. Interestingly, among the two samples analysed for anti-inflammatory activity, *in-vitro* root tuber extract exhibited comparably better activity than *in-vivo* extract either due to the stimulatory effect induced under the *in-vitro* conditions due to the effect of plant growth regulators (PGRs) that might have enhanced the production of different secondary metabolites particularly the terpenoid compounds that confer the anti-inflammatory activity.

Conclusion

From the results of present investigation, it can be concluded that the methanol root tuber extract of *H. annulare* possess a strong antioxidant and anti-inflammatory activity that might be attributed to the presence of the sterols, terpenoids and flavonoid contents. These findings also suggest that this plant have a potential source of natural pharmacological agents which might be helpful in preventing the progress of various chronic diseases. The results also emphasizes the potential utilization of *in-vitro* tubers of the species as substitutes for *in-vivo* samples for drug formulations. However, further studies are needed for the isolation and identification of bioactive compounds and its evaluation in *in-vivo* models to understand their exact mechanism of action as an antioxidant and anti-inflammatory agents.

Conflict Of Interest

The authors declare that there is no conflict of interest.

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