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PHYTOCHEMICAL, ANTIOXIDANT AND CYTOTOXIC ACTIVITY OF SOLVENT EXTRACTS OF *HEDYCHIUM SPICATUM* (LEAF)

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ABSTRACT
Hedychium spicatum is a rhizomatous crop belonging to family Zingiberaceae. Solvent leaf extracts of the plant were explored for phytochemical analysis, cytotoxic and antioxidant activity. Four solvent extracts were prepared these were hexane, chloroform, ethyl acetate and methanol extracts. Leaf extracts exhibited the presence of secondary metabolites like alkaloids, terpenoids and tannins. Methanol extract was rich in phenolics content. Antioxidant activity of ethyl acetate and methanol extract was comparable to the standard Ascorbic acid. However, extracts showed mild cytotoxic activity (<50%).

KEYWORDS: Phytochemical, Antioxidant activity, Brine shrimp assay, DPPH assay.

INTRODUCTION

Hedychium spicatum is a perennial rhizomatous herb belonging to the family Zingiberaceae. Family zingiberaceae consists of 52 genera and over 1500 species and is well known for its medicinal, cosmetics, fragrance and food value.[1] Major commercially cultivated members of the family are *Zingiber officinale* (Ginger), *Curcuma longa* (Turmeric), *Amomum subulatum* (Large cardamom) and *Elettaria cardamomum* (Small cardamom).[2] Family is widely used in treating inflammation, pain, asthma, foul breath, vomiting, diarrhoea, bronchitis, hiccough and blood diseases.[3] *Hedychium spicatum* is commonly known as spiked ginger lily, Van haldi and Kapoorkachari.[4] *Hedychium spicatum's* rhizome is extracted for its aromatic oil which is used as antifungal and antioxidant besides other pharmacological activities.[5] In ayurvedic system of medicines plant is known as shati and is used in a number of polyherbal formulations.[6] Although rhizome of the plant is well known for its medicinal potential, in the present study leaf extracts were explored for their medicinal potential as an alternative source.

MATERIALS AND METHODS

Plant Collection and Preparation of Solvent extract

Fresh leaves of *Hedychium spicatum* were collected from the medicinal germplasm garden of Regional plant resource center (RPRC), Bhubaneswar. Leaves were weighed and were washed with running tap water to remove dust and impurities. After drying, weight of leaves was again taken for the determination of Moisture content. Moisture content of the leaves was calculated by using the following formula

$$\text{Moisture content (\%)} = \frac{Fw - Dw}{Fw} \times 100$$

Where, Fw = Fresh weight of leaf sample
Dw = Dry weight of powdered leaf sample

Further, Leaves were shade dried for about 1 week followed by grinding in grinder (Lexus make) to make fine powder for the preparation of solvent extracts.

Solvent extraction was done by using Soxhlet extraction method. 63.28gm of leaf powder of *Hedychium spicatum* was taken in a thimble made up of cellulose and was subjected to serial extraction with different solvents like Hexane, Chloroform, Ethyl acetate and Methanol on the basis of their increasing polarity. 400ml of solvent was taken in a round bottomed flask and refluxed continuously for 2-3 days so as to get the maximum number of soluble molecules in a particular solvent. After extraction the extract was concentrated by using Buchhi(R-200) Rotavapour under vacuum at 45-50°C depending upon the boiling point of the solvent. Yield of the solvent extracts was also recorded.

Concentrated extracts were transferred to screw cap vials and extract yield was calculated by using the formula-

$$\textbf{Percentage yield of extract} = \frac{\textbf{\textit{Extract weight}} \times \textbf{100}}{\textbf{\textit{Powdered weight}}}$$

Phytochemical analysis

Phytochemical analysis was conducted following the standard protocols.[7] Brief description of various tests conducted for different metabolites is as follows

1. **Test for alkaloids:** Alkaloids tests were done by using 3 different reagents.
- **Dragendroff' s test** –To 1ml of extract 2ml of 1%HCL was added and boiled for few minutes after boiling 2 -3 drops of Dragendroff ‘s reagent was added and sample was observed for reddish brown precipitate .
- **Wagner's test-**To 100µl of extract 2ml of diluted HCl was added followed by 1ml of Wagner's Reagents drop wise. Formation of reddish brown precipitate indicates the presence of alkaloids.
- **Mayer's test** - To 100µl of methanolic extract 2ml of diluted HCl and 1ml of Mayer's Reagent was added. Formation of yellow cream colour represents the presence of alkaloids.

2. **Test for flavonoids:** To1 ml of methanolic extract 1ml of 10% NaOH was added from the side of the tube, drops of conc. HCL was added. Yellow colour turns to colourless which indicates presence of flavonoids.
3. **Test for anthraquinone:** To 1ml of extract 2ml of 5% KOH was added and was observed for pink colouration.
4. **Test for saponin:** To 1ml of extract 2ml of NaHCO3 was added which on shaking forms lather if saponin is present.
5. **Test for terpenoids:** To 1ml of extract, 400 microlitre of chloroform and 4-5 drops of conc.H_2SO_4 was added from the wall of the test tube. Reddish brown precipitate indicates the presence of terpenoids.
6. **Test for cardiac glycosides:** To 5ml of extract 2ml of glacial acetic acid, few drop of $FeCl_3$ and conc.H_2SO_4 was added from the wall of the test tube. Presence of cardiac glycosides is determined by Reddish brown ring.
7. **Test for tannin:** It can be observed by 2 methods.
- 1ml of extract was boiled and few drops of $FeCl_3$ were added to it. The sample was observed for blue, black and green colour.
- To 1ml of extract 500 microliter of lead acetate was added which gives yellow colour.

8. **Test for Phlobotanin:** To 1ml of extract 1% HCl was added and boiled, formation of precipitation occurred on positive test.

Determination of total phenolic content

The total phenolic content (TPC) of the leaf extract was determined using the method of Singleton *et al.*[8] First standard was calculated by using Gallic acid and then leaf extract of different solvents i.e. hexane, chloroform, ethyl acetate, methanol were used.

Concentrations of 1mm, 0.5mm, 0.25mm, 0.125mm, 0.062mm, 0.031mm of gallic acid and plant extracts were prepared in methanol. To 0.5 ml of test sample (gallic acid or leaf extract), 1.5ml (1 : 10 v/v diluted with distilled water) FolinCiocalteau´s reagent was added and allowed to stand for 5 min at 22˚C. After 5 min, 2.0 ml of 7.5% of sodium carbonate was added. These mixtures were incubated for 90 min in the dark after slight shaking. After incubation development of blue colour was observed. Absorbance of different samples was measured at 725nm using spectrophotometer. The phenolic content was calculated as gallic acid equivalents GAE / g on the basis of standard curve of gallic acid. All the experiments were carried out three times.

Antioxidant activity

Qualitative analysis (TLC based antioxidant assay)

TLC is one of the most widely used and potent techniques to resolve mixture of plant compounds. It is also called DPPH (2, 2- diphenyl – 1 – picrylhydrazyl) assay.[9] The TLC sheets supplied by merck, Germany (TLC silica gel 60 F 254) was used as stationary phase. The developed TLC was sprayed with 0.2% DPPH in methanol as indicator as per the standard protocol.[9] The presence of antioxidant compounds detected by yellow spots against purple background on the TLC sheet.

DPPH + AH → DPPH - H + A^-
(violet colour) (yellow colour)

Three types of solvents were prepared for TLC chromatography techniques.

BEA -Benzene: Ethanol: Ammonium hydroxide (90: 10: 1)[Non polar / Basic]

EMW -Ethyl acetate: Methanol: water (40: 5.4: 4 [Polar / neutral]

CEF - Chloroform: Ethyl acetate: Formic acid (5: 4: 1) [Acidic]

Qualitative screenings of the constituents in each of the plant extracts of *Hedychium spicatum* for antioxidant activity was done by TLC based antioxidant assay. The silica coated TLC sheet was activated at 100degree for 2 minutes. The sample were loaded on the TLC sheet with the help of micro tips by leaving 1cm from the bottom of the sheet. Chromatogram was run upto 8cms in the three different solvents as mentioned above. After drying the DPPH solution was sprayed on the TLC sheet.

Rf values of all the antioxidant bands were calculated using the following formulae.

Retardation factor = Distance travelled by the extract
Total distance travelled by the solvent

Quantitative anti-oxidant analysis

Quantitative analysis was done by two popular methods as follows;

DPPH free radical scavenging assay

For DPPH free radical scavenging assay[10] 1mM DPPH (2, 2-diphenyl-1-picrylhydrazyl) solution was prepared by adding 4mg of DPPH dissolved in 10ml methanol. DPPH assay was done by serial dilution method starting from concentration of plant extracts (7.8µg/ml, 15.62µg/ml, 31.25µg/ml, 62.5µg/ml, 125µg/ml,

250µg/ml, 500µg/ml, 1000µg/ml) was prepared in methanol. 1ml of each sample was taken in the test tubes and 500µl of DPPH solution was added. For control, each test tube contained 1ml methanol and 500µl DPPH**.** Samples were incubated for 30 minutes at room temperature in dark. All the samples were taken in triplicate and complete set of experiment was repeated three times. Optical density (OD) was measured at 517nm in spectrophotometer. The percentage of free radical scavenging activity was calculated from the following formula.

Percentage free radical scavenging [DPPH] = [(Ac – As) ÷ Ac] × 100

Where, Ac = Absorbance of control and As = Absorbance of sample.

FRAP Assay (Estimation of total antioxidant activity)

Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay as per the standard protocols.[11] Spectroscopic method is based upon the ability of antioxidants to reduce Fe+3 to Fe+2 in the presence of TPTZ, forming an intense blue Fe+2-TPTZ complex with an absorption maximum at 593 nm. The decrease in absorbance is proportional to the antioxidant present. The FRAP reagent (300 mM Acetate buffer pH 3.6: 40 mM Dilute HCl: 10 mM TPTZ: 20 mM FeCl3.6H2O in the ratio of 10: 1: 1) was prepared and then incubated at 37oC in a water bath for 10 minutes. Absorbance of FRAP reagent was taken at 0th minute (t0) which was the control of the experiment. Ascorbic acid was taken as standard. A total of 100 µL of sample/standard and 300µl of distilled H2O was then added to the FRAP reagent and incubated at 37°C for 4 minutes. A reagent blank was prepared as described above but 100µl of distilled H2O was added instead of test sample. Duplicate test tubes were taken and absorbance was measured at 593nm. Ascorbic acid was taken as standard and 1.0mM to 0.1mM concentration of standard was prepared for the FRAP assay and based on the observations a standard curve was plotted. A number of dilutions of each sample extract were tested allowing dose response curves to be produced.

Cytotoxic activity

Brine shrimp *(Artemia salina*) mortality assay

Cytotoxic activity study was carried out by brine shrimp lethality assay using standard protocols.[12] Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. Brine shrimp (*Artemia salina*) eggs were hatched in artificial sea water, which was prepared using black salt 2 gm/ 200 ml distilled water. The eggs were incubated for 24 hours at temperature of about 28° C to get the desired growth of the larvae for biological evaluation. For each dose level 3 replicates were used. To each test tube of control, positive control and extracts, 20 numbers of brine shrimp and volume was made up to 10ml by adding salt water. Cytotoxic assay was carried out at three doses 500, 1000 and 2000µg/ml. Motility assessment of larvae was conducted at each hour up to four hours.

Motility readings were graded as below.

4+ = high motile
3+ = motile
2+ = sluggish
1+ = slow
Nil = no activity

After 24 hrs, the number of survived larvae in the control and experimental tubes were counted. From this data, the percentage (%) of inhibition of the brine shrimp was calculated for each concentration using the following formula

% Inhibition = No of larvae (Control) – No. Of larvae (Experiment) X100

No. of larvae in control

RESULTS AND DISCUSSION

Moisture content of leaves was found to be 88.36 percent and as can be observed from Table 1 Yield of methanol extract was highest followed by chloroform extract. As methanol extract showed yield higher than all extracts together suggests the prominent presence of polar molecules in the plant.

Table 1: Yield of solvent extracts of *Hedychium spicatum* leaf.

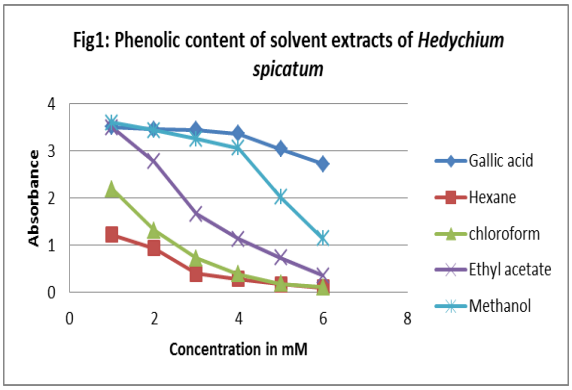
Solvent extracts	Percentage Yield
Hexane	4.58
Chloroform	5.76
Ethyl acetate	2.34
Methanol	19.2

Phytochemical screening of the plant material revealed the presence of alkaloids and tannins. The tannins have anti proliferative and apoptotic effects against a variety of compounds and protect the plants from being infected by bacteria or fungi.[13] All the tests for alkaloids were positive in all the solvents. Methanol extract showed the presence of terpenoids and cardiac glycosides as well. Terpenoids are used by plants as primary as well as secondary metabolite, as primary metabolite it provides support in the growth of plants and as secondary metabolites it acts as protective agent for plants.[14] Cardiac glycosides are considered as potent anticancer agents[15] as they target senescent cells in the body. Thus, phytochemical analysis of leaf extracts has provided a lead that leaves of the plant are also rich in medicinal potential.

Table 2: Phytochemical screening of leaf extract of *Hedychium spicatum*.

Secondary metabolites	Hexane	Chloroform	Ethyl acetate	Methanol
Alkaloids	+	+	+	+
Mayer's Test	+	+	+	+
Wagner's Test	+	+	+	+
Dragendroff's Test	+	+	+	+
Flavonoids	-	-	-	-
Anthraquinone	-	-	-	-
Saponin	-	-	-	-
Tannin	+	+	+	+
Terpenoids	-	-	-	+
Phlabotanins	-	-	-	-
Cardiac glycoside	-	-	-	+

Total phenolic content of leaf solvent extracts of *Hedychium spicatum* was determined using Gallic acid as standard. As can be seen from Figure 1, at higher concentration methanol extract exhibited similar phenolics content as the standard molecule. The hydroxyl groups in plant extracts are responsible for facilitating free radical scavenging.[16]



Anti-oxidant activity of *hedychium spicatum*

Qualitative antioxidant screening

As per the standard protocol, all the extracts were run in three different solvents i.e. CEF, BEA and EMW. DPPH is reduced and resulting molecule gives a yellow band when chromatograph is sprayed with 0.2% DPPH solution. Best separation was obtained in BEA solvent where bands all the extracts showed maximum number of antioxidant activity with maximum number of 8 bands in the chloroform extract. Number of bands is directly correlated with the extent of antioxidant activity of the extract.

Table 3: TLC based DPPH ASSAY of solvent extracts of *Hedychium spicatum*.

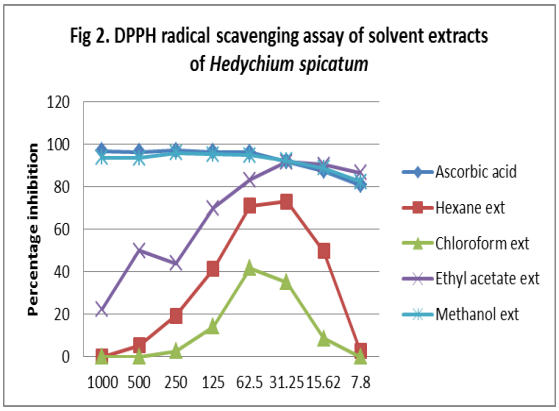
Solvent extract	Solvent system	No. of bands	Rf values
Hexane	BEA	0	0
	CEF	4	0.21, 0.23, 0.25, 0.42
	EMW	0	0
Chloroform	BEA	3	0.21, 0.24, 0.4
	CEF	3	0.7, 0.75, 0.78
	EMW	2	0.13, 0.31
Ethyl acetate	BEA	0	0
	CEF	3	0.42, 0.52, 0.7
	EMW	0	0
Methanol	BEA	0	0
	CEF	2	0.46, 0.55
	EMW	0	0

Quantitative antioxidant activity

TLC based DPPH Assay

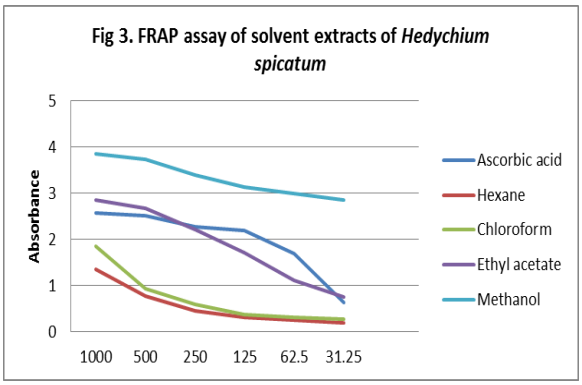
The reactivity of different extract of *Hedychium spicatum* was analysed with DPPH, a stable free radical. As DPPH picks up one electron in the presence of free radical scavenger, the absorption decreased and the resulting discoloration was related to the number of electrons gained. The DPPH free radical scavenging activity of different extract (Hexane, Chloroform, Ethyl acetate, Methanol) was determined by spectoroscopic

assay at 517nm. *Hedychium spicatum* hexane extract exerted an inhibition of 73.26% at 31.25µl/ml, chloroform extract exerted an inhibition 41.75% at 62.5µl/ml, ethyl acetate extract exerted an inhibition of 91.75% at 31.25µl/ml, methanol extract exerted an inhibition of 95.99% at 250µl/ml and that of ascorbic acid was 97.09% at 250µl/ml. Methanol extract's activity was similar to the standard molecule ascorbic acid.



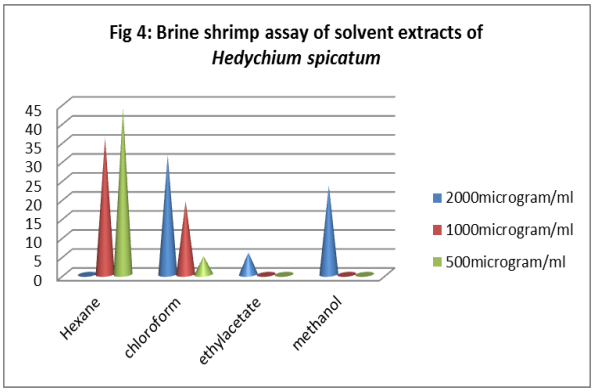
Ferric reducing antioxidant power assay

The antioxidant can donate an electron to free radicals, which leads to neutralization of the radical. Reducing power was measured by direct electron donation in the reduction of Fe3+ to Fe2+ in the presence of TPTZ. The product was visualized by forming an intense blue colour complex and then measured at 593nm. As can be seen in the Fig 3, none of the extracts showed similar activity when compared with the standard molecule ascorbic acid. All the extract showed mild activity.



Cytotoxic activity of solvent extracts of Hedychium spicatum

All the extracts showed mild activity as can be seen from Fig 5.



Overall it can be concluded that methanol extract of the plant holds promise as a potential antioxidant candidate.

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