PRELIMINARY PHYTOCHEMICAL SCREENING, CHEMICAL INVESTIGATION, ENZYME INHIBITING ACTIVITY AND ATOMIC ABSORPTION SPECTROPHOTOMETRIC DETERMINATION OF MINERALS OF PLANT EXTRACTS OF *SCOPARIA DULCIS*.LINN.

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ABSTRACT

The phytochemical studies on the plant of *Scoparia dulcis*.Linn resulted in isolation of α and β amyrin and steroidal glycoside are being reported for the first time from this plants. The preliminary phyto chemical screening showed the presence of Carbohydrates, protein and aminoacids, glycosides, phenolic compounds, steroids, di and tri terpenes, and flavanoids. The plant is reported to contain flavonoids, which was evident from the phytochemical investigation of crude extract, an attempt was made to identify these flavonoids by paper chromatography. It is stated that phenols readily complex with protein and as a result there is often inhibition of enzyme activity. Since this plant contains phenolic derivatives an attempt was made for the possible enzyme inhibitory action. The alcohol and aqueous extract exhibited high degree of inhibition towards the enzymes tested. These compounds have been characterized on the basis of spectral and other data. The isolated compounds (BJ I, BJ II, BJ III, BJ IV and BJ V) from column chromatography. An attempt to estimate the amount of magnesium present in the plant by atomic absorption spectrophotometry, was made and the presence of an appreciable amount of magnesium was established. The ash content of the sample was 7% and the magnesium concentration was found to be 41.342mg/L. that is the percentage of magnesium content in the air dried crude drug is 0.567%.

Keywords :Phyto chemical, *Mimosa pudica*, Enzyme inhibitory activity , IR, NMR

INTRODUCTION

Scoparia dulcis.Linn (Sweet Broomweed) Family Scrophulariaceae. Indigenous to tropical America and introduced to India; commonly found as a weed in many parts of India, particularly in West Bengal, Karnataka, Tamilnadu and kerala. A tough, glabrous, leafy-branched, herbaceous plant upto 90cm high, leaves are opposite or whorled, lanceolate, coarsely serrate, 0.5-2cm long. Flowers are small, white, in small 2-4or 5 flowered inflorescences, capsules glabrous, 3-4mm in diameter.[1,2,3]An infusion of the leaf is used in fever, cough and bronchitis and as gargle for toothache. A hot infusion is a diuretic. An infusion of roots leaves and tops are useful in diarrhea and dysentery. All parts of the plant are useful as emetic. An infusion of seeds obtained by soaking them in water overnight is a cooling drink. *Scoparia dulcis* is medicinally used in Paraguay as crude drug namely "Typycha Kuratu" to improve digestion and protect the stomach. In Taiwan, the same plant is used as cure for hypertension and in India for toothache, blennorhagia and stomach troubles. An anti-diabetic compound, Amellin, has been reported in the leaf and stem of fresh green plant.[4,5,6,7,8]. Leaves of *Scoparia dulcis*. Linn were reported to contain flavonoids scutellarein, 7-o-methyl Scutellarein and Scutellarin.[9] A flavone cirsitakaoside was extracted from *Scoparia dulcis*.Linn.[10]An ethanolic extract of *Scoparia dulcis*.Linn wa found to exhibit sympathomimetic activity. The herb *Scoparia dulcis*.Linn is used in Brazilian folk medicine to treat bronchitis, gastric disorders, hemorrhoids, insect bites and skin wounds, and in oriental medicine to treat hypertension.[11] The water and ethanolic extracts of *Scoparia dulcis* Linn was found to possess analgesic, anti-inflammatory and antipyretic activities.[12] Scopadulin a novel tetracyclic diterpine from *Scoparia dulcis*.Linn was found to possess anti viral properties.[13] The effect of acacetin isolated from *Scoparia dulcis*.Linn and several related flavonoids on herpes simplex virus type-I was studied in vitro by the method of plaque yield reduction.[14] Transgenic herbicide resistant *Scoparia dulcis*.Linn plants were obtained by using refinery vector system.[15,16]

MATERIALS AND METHODS

Plant material

The plants were collected from the watery areas of Thrissur district, Kerala in the month of June. This was identified as *Scoparia dulcis* Linn of Scrophulariaceae family by Dr.Stephen, American College, Madurai.

Preparation of extracts

500 grams of the dried coarse powder of *Scoparia dulcis* Linn, was packed in a Soxhlet apparatus and was extracted by hot percolation method using different solvents namely pet ether (60-80), benzene, chloroform, ethanol and water. The extraction was carried out with 750ml of each solvent for a period of 24 hours. At the end of the extr4eaction the respective solvents were concentrated under reduced pressure and the crude extracts were stored in desiccators.

The marc left was then dried and extracted with the next solvent.

Preparation of column isolated compounds

30 grams of the benzene residue was dissolved in 60ml of acetone. Then it was added to 50grams of silica gel (for column chromatography, 60-120mesh) with constant stirring. This facilitated the evaporation of the solvent to get gel impregnated with the above fraction of benzene.

Into the glass column of 1.5 meters length and 3.5 cm diameter the suspension of silica gel (for column chromatography 60-120 mesh) 300grams in n-hexane was packed uniformly. Then the silica gel impregnated with the sample was packed uniformly on top of the silica gel column and was eluted with different proportions of n-hexane, benzene, chloroform, ethyl acetate and little methanol. The elution was carried out at the rate of 30 drops per minutes. Totally 160 fractions each of 200ml were collected. Each of the fractions were distilled and concentrated and were collected in a separate containers.

Fraction 45-55 (eluted with n-hexane and increasing amount of benzene up to 100%) were collected and yielded a colourless solid on distillation. They were mixed together and concentrated. This left a colourless compound. This was recrystallized from methanol and n-hexane, which yielded a colourless compound (600mg), which was found to be homogeneous on T.L.C. and showed a melting point 198-200°C. This compound was designated as BJ-I.

Fractions 56-63 (eluted with benzene and increasing amount of chloroform up to 20%) yielded a light green coloured residue on distillation which was a mixture of two compounds, which on recrystalisation from methanol yielded a colourless compound (60mg). This was found to be homogeneous on T.L.C. and showed a melting point 170-172°C. This compound was designated as BJ-II.

Fraction 69-87 (eluted with benzene and increasing amount of chloroform up to 100%) yielded a green coloured residue. They were mixed together and concentrated and was recrystallized from methanol and n-hexane to yield a light green coloured compound (220mg).This was found to be homogeneous on T.L.C. and showed a melting point 88-92°C. This compound was designated as BJ-III.

Fractions 88-104 (eluted with benzene and increasing amount of chloroform up to 70%) yielded a green coloured mass. They were mixed together and concentrated and was recrystallized from n-hexane to yield a green coloured compound (240mg)of the compound. This was found to be homogeneous on T.L.C. and showed a melting point 132-136°C. This compound was designated as BJ-IV.

Fractions 121-134 (eluted with chloroform and increasing amount of ethyl acetate up to 100%) yielded a green coloured compound on removal of the solvent. They were mixed together and concentrated and was recrystallized from n-hexane to yield a green coloured compound (380mg) of the compound. This was found to be homogeneous on T.L.C. and showed a melting point 30-302°C. This compound was designated as BJ-V. This was later hydrolyzed with 2N hydrochloric acid for 1 hour and was tested for the presence of sugars.

Preliminary phytochemical screening [17,18,19,20]

Carbohydrates, protein and aminoacids, glycosides, phenolic compounds, steroids, di and tri terpenes, and flavanoids were present in the extracts.

Enzyme inhibitor activity[21,22,23]

Preparation of the enzyme

Salivary amylase

10ml of the saliva was collected and diluted to 100ml with cold phosphate buffer pH 7.1. The solution was centrifuged at 8000 rpm for 20mts and the clear supernatant was used.

Extraction of crude inhibitor

There are several reports on the extraction of enzyme inhibitors from plant materials. We have adopted the method Buonocore et.al 100mgs of dried plant powder was extracted with 2.5% of cold TCA with magnetic stirrer for 45mts. The solution was centrifuged to get a clear supernatant, which was neutralized to pH 7 with dilute sodium carbonate and used for the assay of enzyme inhibition. This is referred as the crude inhibitor.

Amylase assay

Amylase activity was determined by the method of Bernfeld 1ml of the enzyme solution was added to 2ml of phosphate buffer pH6.9 containing 2MNacl and the reaction started with the addition of 2ml 1% soluble starch solution. The tubes were incubated at 37°C fir 20mts. The reaction was arrested by the addition of 1ml for dinitrosalicylic acid colour reagent. The tubes were kept in a boiling water bath for 10mts, cooled and diluted t a final volume of 10ml with distilled water. The absorbance was measured at 530nm in an Erma photoelectric colorimeter. Amylolytic activity was calculated as maltose equivalents liberated. One unit of enzyme is defined as the milligrams of maltose liberated per minutes under the assay conditions. The standard curve was constructed using pure anhydrous maltose. The assays were run along suitable blank (without enzyme).

To measure the amylase inhibitory activity, suitable amount of 0.1% mercuric chloride was pre incubated with the enzyme, and the assay was carried out as described above. The inhibition caused by 0.5% of mercuric chloride was arbitrarily fixed as 100% inhibition. Suitable amounts of plant extract were similarly tested for the presence of amylase inhibitors in the extract. The decrease in the inhibitory activity of the crude plant extract was defined on the basis of these parameters. The crude extracts showed about 80-100% inhibition.

$$\%\ \text{inhibition} = \frac{\text{Abs of control (no inhibitor)} - \text{Abs of sample}}{\text{Abs of control (no inhibitor)}} \times 100$$

The crude extracts inhibitory activity was not destroyed when heated to 80°C, thus revealing its likely non proteinaceous nature. From these observations it can be

inferred that further purification can lead to more information on these natural enzyme inhibitors.

Assay of Urease

Urease enzyme was prepared from horse gram seeds. The assay of enzyme was carried out according to the method described by Malhothra using Nessler's reagent. A standard was prepared using NH_4SO_4.urease inhibition was compared with parachloromercuric benzoate (PCMB), the activity of which was set arbitrarily to 100%. Under these conditions the crude extract showed about 100% of inhibition.

Assay of Trypsin

Commercial trypsin units obtained from sodium dodecyl sulfate (SDS) and was assayed according to the method of Kunitz et, al using casein as substrate. The extracts showed about 50-60% inhibition when compared with purified trypsin inhibitors, which was arbitrarily fixed as 100%[24]

Atomic absorption sepectrometric determination of minerals

Atomic absorption spectrophotometry is the determination of concentration of a substance by the measurement of absorption of the characteristic radiation by the atomic vapour of as element, when radiation characteristic of a particular elements passes through an atomic vapour of the same element, absorption of radiation occur in proportion to the concentration of the atoms in the light path. When sample size is limited and higher degree of sensitivity is desired atomic absorption spectrophotometry is used [25,26]

Ashing of sample

About 2gm of sample was weighed accurately in a quartz crucible (which was previously been heated to about 600°C and cooled). The crucible is placed on a clay pipe triangle and heated first over a low flame till all the material is completely charred, followed by heating in a muffle furnace for about 3-4hours at about 6oo°C, it was then cooled in a desiccators and weighed to ensure complete ashing, the crucible was again heated in the muffle-furnace for half an hour, cooled and weighed. This was repeated till two consecutive weights are the same and ash was almost white or grayish white in colour.

Ash content % = weight of the ash/weight of the sample taken x 100

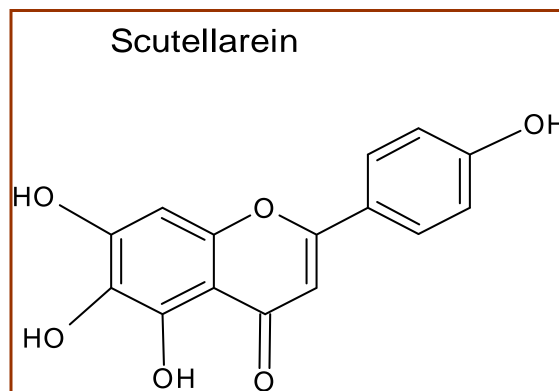
140mg/2000mg x 100 = 7%

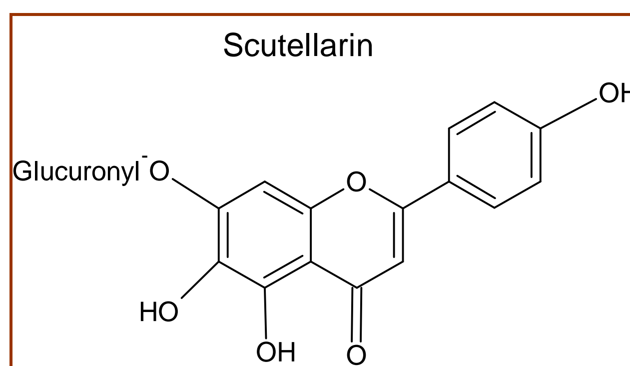
Ash solution

The ash is moistened with a small amount of glass distilled water (0.5-1.0ml) and 5 ml; of distilled hydrochloric acid is added to it. The mixture is evaporated to dryness on a boiling water bath. Another 5ml of hydrochloric chloride is added again and the solution evaporated to dryness as before. Four ml of 0.1N Hydrochloric acid and a few ml of water are then added and the solution warmed over a boiling water bath and filter into a 250ml volumetric flask using Whatman No 40 filter paper. After cooling the volume is made up to 25ml and was used for the estimation of metals.

Result from the atomic absorption spectrophotometric studies the magnesium content in the sample was found to be 41.342mg/ml. the percentage of magnesium content in the air dried crude drug was found to be 0.567%.

Phytochemical investigation by paper chromatography[27]





The marc obtained after benzene extraction were well dried and was extracted with aqueous alcohol (5lit) for 72 hours. This was then filtered hot and filtrate (4lit) were concentrated by vacuum evaporations, to give 55 grams of residue which was dark brown in colour.20 grams of the residue was dissolved in water and it was filtered. Them the filtrate was partitioned with ether (4x100ml) in a separating funnel to obtain the ether extract and aqueous extract. The ether extract was then concentrated and spotted on Whatman filter paper. The aqueous extract was then partitioned with ethyl acetate (4x100) to obtain the ethyl acetate fraction and the aqueous fraction. The ethyl acetate fraction was concentrated and was used for spotting on Whatman filter paper. The aqueous fraction which remained was also screened.

Preparation of solvent systems

Various solvent systems namely distilled water, 5,15,30,50,and 60% acetic acid, Butanol: aceticacid: water(4:1:5) Seikel and phenol were prepared by shaking mixtures of solvents together in appropriate proportions in a separating funnel, shaking the mixture well and allow in git to separate whenever necessary into the organic and aqueous phases. The two layers were separated and stored separately. When there was no separation into two layers, the whole mixture was used for both equilibrations of the chamber and to develop the paper.

Equilibration of the systems

The solvents were placed in the trough. It was left for 12-24 hours for equilibration closing the chamber air tight to avoid escape of solvent vapors.

Development of chromatogram

After equilibration, the spotted paper strip was hung in the air-tight chamber. The lower end of the paper strip was immersed in a trough containing the solvent. The lid was replaced immediately. The development was carried out until the solvent front had moved up to 15-16cm. the chromatogram was taken out, the solvent front marked and then dried at room temperature.

Location of the spot

The position of the spots were marked either by observing in short or long wave ultraviolet, which showed a pink fluorescence and the fluorescence was identified by exposing the paper to ammonia vapor. The spots were marked and the Rf values were calculated and were compared with the values recorded for the standard compound. The values obtained and the compounds identified are shown in the table.

Table 1: Activities of some enzyme and enzyme inhibitors in extracts of *Scoparia dulcis* linn

Enzyme/enzyme inhibitor	Standard inhibitor	Extracts (percentage of inhibition)					
		1	2	3	4	5	6
Amylase	Mercuric chloride	100%	-	-	Trace	80%	100%
Urease	p-chloro mercuric benzoate	100%	-	-	Trace	100%	100%
Trypsin	Jack bean crude inhibitor	100%	-	-	Trace	60%	50%

1-standard inhibition, 2-pet ether extract, 3-benzene extract, 4-chloroform extract, 5-alcohol extract, 6-aqueous extract

Table 2: Rf values of the flavonoids of *Scoparia dulcis* Linn [Rf x100 (Whatman No.1 ascendfing)]

Compound	Developing solvents								
	A	B	C	D	E	F	G	H	I
Scutellarein	2	3	7	33	42	50	85	86	76
Scutellarin	50	13	20	42	56	60	34	57	50

A-distilled water, B-5% aqueous acetic acid, C-15% aqueous acetic acid, D-30% aqueous acetic acid, E-50% aqueous acetic acid, F-60% aqueous acetic acid, G-n-Butanol:Aceticacid:water (4:1:5)v/v upper layer used, H-Seikel (27% aceticacid and 73% n-Butanol v/v, upper layer used), I-Phenol saturated with water (lower layer used).

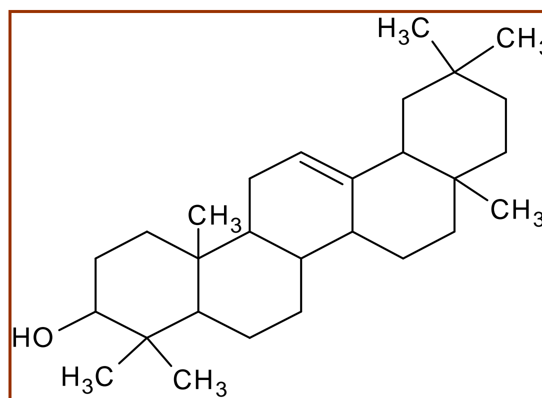
Spots were visualized under UV lamp. (Camag UV light 254nm and 366 nm)

Phytochemical investigation by column chromatography isolated compound [28,29,30]

Compound BJ-I crystallized from chloroform-n-hexane as colourless needles, showing a melting point 198-200°C. it gave a pink colour with Leiberman_Burchard test and a yellow colour with tetranitomethane, indicating it to be triperpenoid[31]. It's I.R. spectrum revealed the presence of a hydroxyl function (3400cm^{-1}), which is supported by a multiplet at δ3.47 in the PMR spectrum for a 3 α-hydrogen.

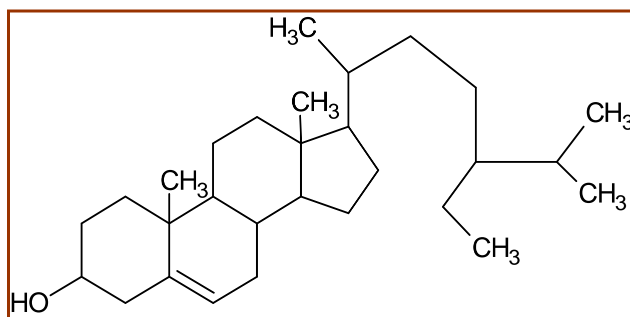
The PMR spectrum of the compound showed eight singlets in the region δ0.1-1.2, which was assigned to eight aliphatic methyls. The presence of Δ^{12}, double bond is revealed by a multiplet at δ5.6 and an unresolved doublet at δ3.4 for an allylic proton (H-18). These assignments were further supported by the ^{13}C-NMR spectrum of the compounds, which showed signals at δ121.5(C-12) and δ140.9(C-14). From the ^{13}C chemical shift of C-18 (δ46.7),C-19 (42.38) and C-20(31.7), the compound BJ-I has been tentatively identified as β-amyrin.

Compound BJ-II crystallized from methanol as colourless crystals, showing a melting point 170-172°C. It gave a pink colour with Leiberman_Burchard test. It's I.R. spectrum revealed the presence of a hydroxyl function (3400cm^{-1}) and it's PMR and ^{13}C NMR are suggestive of those a triterpenoid. However the NMR did not agree either with that of α or β amyrin, due to paucity of the material further characterization could not be carried out.



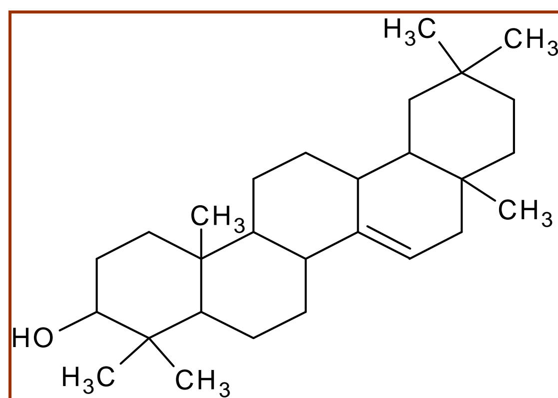
Compound BJ-III Crystallized from methanol-n-hexane as green flakes, melted at 88-92°C. It did not answer with Leiberman_Burchard test. Although It's I.R. spectrum showed a band at 1680cm^{-1}, it's ^{13}CNMR revealed the absence of unsaturation and a carbonyl function. In order to account for it's ^{13}CNMR spectral data the following tentative aliphatic ether structure was proposed and is also consistent with reported melting point.

Compound BJ-IV



Crystallized from n-hexane as light green coloured needles, melted at 132-136C. it gave a green colour with Lieberman-Burchard test. Based on its spectral data (PMR and 13CNMR) the compound was identified a β-sitosterol, and its identity was further confirmed by direct comparison (co-TLC) with an authentic sample generously provided by Dr.P.Ramesh, Department of Natural Product Chemistry, School of Chemistry, M.K.University.

Compound BJ-V



Crystallized from n-hexane as green coloured needles, melted at 300-302°C. It gave a green colour in Leiberman_Burchard test and a positive Molicsh test, on hydrolysis with 2N hydrochloric acid. Its I.R. spectrum revealed the presence of a hydroxyl function (3420cm^{-1}) and a carbonyl function (1660cm^{-1}). The PMR spectrum of the compound revealed at least six aliphatic methyl and the presence of unsaturaion is indicated by a multiplet at δ5.37, a multiplet centered at δ3.0 was assigned to 3α-hydrogen, while a well resolved downfield doublet at δ4.5 was assumed to be due to anomeric proton of the glycoside. This assumption was supported by the presence of another multiplet centered at δ3.18 which was assigned to the remaining sugar protons based on the above data, the compound BJ–V was tentatively identified as a steroidal glycoside.

RESULTS AND DISCUSSION

The preliminary phytochemical screening showed the presence of Carbohydrates, protein and aminoacids, glycosides, phenolic compounds, steroids, di and tri terpenes, and flavanoids. Since the plant is reported to contain flavonoids, which was evident from the phytochemical investigation of crude extract, an attempt was made to identify these flavonoids by paper chromatography. The marc left after benzene extraction was extensively extracted with aqueous alcohol. The residue obtained after extraction was concentrated by centrifugal vacuum evaporation was partitioned with different solvents. These filtrates were concentrated and were subjected to paper chromatography, using nine different solvent systems. By comparing the Rf values obtained with that of standard Rf value, the flavonoids present in the plant were identified as Scutellarein and Scutellarin. It is stated that phenols readily complex with protein and as a result there is often inhibition of enzyme activity. Since this plant contains phenolic derivatives an attempt was made for the possible enzyme inhibitory action.

The alcohol and aqueous extract exhibited high degree of inhibition towards the enzymes tested. Therefore this plant can be a potential source for inhibitors of medically important enzymes. The tissues most likely to be damaged by chronic magnesium depletion are cardiovascular, renal and neuromuscular. It has also been stated that magnesium is an inhibitor of calcium oxalate crystallization and magnesium hydroxide therapy is found to be effective in the treatment of kidney stones. Based on the facts an attempt to estimate the amount of magnesium present in the plant by atomic absorption spectrophotometry, was made and the presence of an appreciable amount of magnesium was established. The ash content of the sample was 7% and the magnesium concentration was found to be 41.342mg/L. that is the percentage of magnesium content in the air dried crude drug is 0.567%.

The melting point of the isolated compounds was found out by open capillary tube method and the results were uncorrected. The purity of the compounds was checked by TLC using silica gel G as an adsorbent, ethyl acetate and chloroform (9:1) were used as mobile phase. The spot was visualized by iodine vapour or dinitrophenyl hydrazine solution. The structure of the isolated compounds was characterized by its IR, HNMR spectral analysis in which it complies with the normal values.

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