IJPSR (2012), Vol. 3, Issue 05 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 20 January, 2012; received in revised form 23 February, 2012; accepted 23 April, 2012

CHARACTERISATION OF BIOACTIVE COMPOUNDS IN *COSTUS SPECIOSUS* (KOEN). BY REVERSE PHASE HPLC

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Keywords:
Costus speciosus,
bioactive compounds,
phenolics,
proteins,
Zingiberaceae

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ABSTRACT

Costus speciosus belongs to the family *Zingiberaceae* and contains lots of medicinal properties. The aim of this study was to screen, characterise and quantify the selected biomolecules *viz*. phenolics, proteins in *C. speciosus*. Ethanol extract possessed high amount of phenolics than the acetone and aqueous extract. Aqueous extract has least amount of phenolics compared to ethanol and acetone extracts. The protein contents were high in phosphate buffer as compared to the aqueous extract. Qualitative estimation of phenolic compounds was accomplished by reverse phase high pressure liquid chromatography. High amount of phenolic content was found in the mature leaf of *C. speciosus*.

INTRODUCTION: Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of wild plants grow in different parts of the country. India is a country rich in indigenous herbal resources which grow on their varied topography and under changing agro- climatic conditions permitting the growth of almost 20,000 plant species, of which about 2,500 are of medicinal value [1].

In Indian scenario, it has been recognized that 2,500 plants have been found to be have medicinal values out of 17,000 plants. The world is now looking towards India for new drugs to manage various challenging diseases because of its rich biodiversity of medicinal plants and abundance of traditional know-how such as Siddha, Ayurveda *etc*., to cure different diseases [2, 3].

Costus speciosus or crape ginger is possibly the best known cultivated species of the genus *Costus*. *Costus speciosus* is an erect herbaceous plant up to 2 m height with long lanceolate leaves and white fragrant flowers in terminal clusters. According to Ayurveda, the rhizomes are bitter, astringent, acrid, cooling, aphrodisiac, purgative, anthelmintic, depurative, febrifuge, expectorant and tonic and useful in burning sensation, constipation, boils, diarrhoea, dizziness, headache, vertigo, ear, eye and nose pain, and to stop vomiting, leprosy, worm infection, skin diseases, fever, rash, intestinal warms, asthma, bronchitis, inflammations, anaemia, stimulant and also used as anti-diabetic [4], snake bites, jaundice, and antibacterial agent.

Many properties of plant products are associated with the presence of phenolic compounds, which are essential for plant development and play an important role in their defence mechanisms. The inclusion of these compounds in the regular diet might be beneficial to human health by lowering incidence of diseases.

Active oxygen molecules such as ($O_2^{\bullet -}$, $OOH^{\bullet}$), hydroxyl ($OH^{\bullet}$) and peroxyl ($ROOH^{\bullet}$) radicals play an important role in oxidative stress related to the pathogenesis of various important diseases. In healthy individuals, the production of free radicals is balanced by anti-oxidative defence system [5].

Different approaches to drug discovery from plants can be enumerated as: random selection followed by chemical screening, random selection followed by one or more biological assays, follow-up of biological activity reports, follow-up of ethnomedical (traditional medicine) use of plants, use of appropriate plant parts as such in powdered form or preparation of enriched/standardized extracts (herbal product development), use of a plant product, biologically potent but beset with other issues, as a lead for further chemistry, and single new compounds as drugs. The approach of herbal drug development is associated with several problems.

The design, determination and implementation of appropriate, clinically relevant, high-throughput bioassays are difficult processes for all drug discovery programmes [6, 7]. The common problem faced during screening of extracts is solubility and the screening of extract libraries is many times problematic, but new techniques including pre-fractionation of extracts can alleviate some of these issues [8, 9]. In recent years, phenolic compounds have been intensively investigated because of their potential health-promoting effects [10-13]. They have been reported to possess many useful properties for human health, including anti-inflammatory, enzyme inhibition, antimicrobial, antiallergic, vascular and cytotoxic antitumor activity, but the most important action of phenolics is their antioxidant activity [14-16].

The reason of investigation of medicinal plants is the ethanobotanic knowledge still existing in remote areas [17]. In present study, plant crude extract was quantitatively analysed by HPLC.

MATERIALS AND METHODS:

Plant collection and establishment: The *Costus speciosus* plantlets were obtained from horticulture station of Tamil Nadu Agricultural University (TNAU), Yercaud and established in the Herbal garden, PRIST University, Thanjavur. The plants were watered at regular intervals and continuously monitored for the growth and development. The leaf samples from the plant were freshly collected at one week intervals for sample processing.

Extraction of protein from *C. speciosus*: The stem/leaf tissues were cut into pieces and immediately plunged in 0.5M phosphate buffer (pH 7) or water. 10.0 to 15.0 ml of buffer for every gram of tissue was used. The tissues were thoroughly macerated in a mortar with pestle for 5 to 10 minutes. This was then passed through two layers of muslin cloth and re-extracted the ground tissues by second grinding. This second extraction ensure complete removal of buffer soluble substances. This second extract passed through muslin cloth. Both the extracts were pooled, filtered through Whatman No. 41 filter paper and the volume was raised with buffer. Protein estimation in this extract was carried out by Lowry's method (1951) [18].

Extraction of phenols from plant tissues using alcohol [19]: The stem/leaf tissues were cut into pieces and immediately plunged in boiling 80% ethyl alcohol and allowed to boil for 5 to 10 minutes. Five to 10.0 ml of alcohol for every gram of tissue was used. The extraction was done on top of a steam bath. After cooling the extract was cooled in a pan of cold water, the tissues were thoroughly crushed in a mortar with pestle for 5 to 10 min. This was then passed through two layers of muslin cloth and re-extracted the ground tissues for 3 minutes in hot 80% alcohol, using 2.0 to 3.0 ml of alcohol for every gram of tissue.

This second extraction ensures complete removal of alcohol soluble substances. Both the extracts were pooled, filtered through Whatman No. 41 filter paper and the volume was raised with 80% ethanol or reduced by rotary evaporation under reduced pressure to represent 5.0 to 10.0 ml of the extract for every gram of tissue used. This extract contains major of the plant secondary metabolites *viz*., reducing and non-reducing sugars, phenols, flavones. The condensate is then stored under refrigerated conditions for carrying out various phytochemical analysis as envisaged.

Estimation of Total Phenols: Estimation of total phenols was carried out by the standardized method of Bray and Thorpe (1954) [20].

Qualitative estimation of Phenolic Compound: Separation of phenolic compounds was accomplished by Shimadzu LC-8A reverse-phase high-pressure liquid chromatography using a Phenomenex C18 column of the dimension 250 x 4.6mm with 5mm particle size. The separation solvents (mobile phase) used were

0.1% phosphoric acid (Solvent A) and 100 % acetonitrile (Solvent B) of HPLC grade. The solvents were first filtered through membrane filter using the Shimadzu solvent filtration kit, so as to remove the particulates very quickly and effectively. The solvents were then degassed for detector stability as air bubbles tend to cause spikes and noise in detectors. The separation of phenolic compounds was programmed with a gradient flow of 5%~95% solvent B and the run was completed in 65 minutes. At the end of each gradient program, Solvent B was decreased to a concentration of 5% over a period of 10 minutes before returning to initial conditions, as given below:

Time (minutes)	Mobile phase
0-0.01	5% Solvent B
0-5	5% Solvent B
5-10	10% Solvent B
10-25	15% Solvent B
25-25	40% Solvent B
45 -55	95% Solvent B
55-65	5% Solvent B

The pump used was binary with maximum pressure set to 300 kgf/cm^2 with a constant flow rate of 1.0 ml/min. Absorbance at 285 nm was monitored by a Photo Diode Array Detector. Peak areas, peak heights and retention times were determined by using the software provided by Shimadzu. The crude plants extracts prepared were then used for HPLC separation. Before injecting, the sample was passed through sample filtration syringe (0.5 mm nylon membrane) to assure complete particulate removal and maximum column life by preventing undesirable contaminants from clogging within the columns.

The phenolic standards (Himedia chemicals) used were cinnamic acid, ferulic acid, vanillic acid, caffeic acid, syringic acid, gallic acid, catechin hydrate, orcinol monohydrate, phloroglucinol, catechol, coumarin and flavone. Retention time of individual standards was determined before creating calibration curves.

100ppm of the mixture of standards were prepared, from which 10, 20, 50 ppm dilutions were made and used for creating standard curves with four concentration points (**Plate 1**). Contents were determined by standard curves and were expressed as parts per million (ppm).

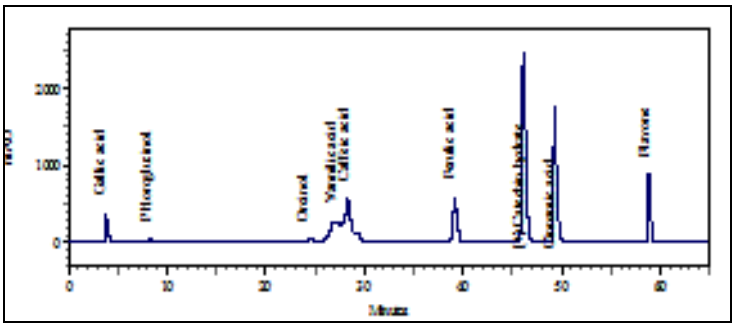

PLATE 1: HPLC CHROMATOGRAM OF PHENOLIC STANDARDS

RESULTS AND DISCUSSION:

Total Protein Contents: The total protein content in different parts of *Costus speciosus* (immature leaf, mature leaf and stem) extracted using phosphate buffer and water were estimated. Total Protein content of the immature leaf of *Costus speciosus* varied from 39.1mg to 55.1 mg per gram tissue. Total Protein content of the mature leaf of *Costus speciosus* varied from 49.1 mg to 71.1 mg per gram tissue. Total Protein content of the stem of *Costus speciosus* varied from 16.6mg to 22.8 mg per gram tissue. The result shows that mature leaf contains high protein value compared to stem and immature leaf. The order of protein content was found to be is mature leaf > immature leaf > stem. The buffer extract was found to contain high amount of protein the water extract (**Table 1**).

TABLE 1: TOTAL PROTEIN CONTENT (mg/g TISSUE) IN *COSTUS SPECIOSUS*

Plant part	Protein content (mg/g tissue)	
	Water	Phosphate buffer(pH 7)
Immature leaf	39.1	55.1
Mature leaf	49.1	71.1
Mature stem	16.6	22.8

Total Phenolic Contents: Total Phenolic content of the immature leaf of *Costus speciosus* varied from 3.7mg to 4.5 mg per gram tissue. Total Phenolic content of the mature leaf of *C. speciosus* varied from 6mg to 14.3 mg per gram tissue. Total phenolic content of the stem of *C. speciosus* varied from 3.2mg to 4.3mg per gram tissue. The study revealed that mature leaf of *C. speciosus* contained high amount of phenolics compared with other two parts of *C. speciosus*. Ethanolic extract has high amount of phenolics than the acetone and water extract. Water extract has least amount of phenolics compared to other two solvents (**Table 2**).

TABLE 2: PHENOLIC CONTENT (MG/G TISSUE) IN *COSTUS SPECIOSUS*

Plant part	Phenolic content (mg/g tissue)		
	80% ethanol	80% acetone	Water
Immature leaf	5.6	4.68	3.7
Mature leaf	14.3	10.42	6.0
Mature stem	4.3	3.50	3.2

Qualitative estimation of Phenolic Compound: The HPLC chromatogram showed that ferulic acid was high (7.878 ppm) compared to other phenolic compound in the acetone extract from mature leaf of *C. Speciosus* (**Plate 2**), Ferulic acid was also high (23.458 ppm) in the ethanolic extract from mature leaf of *C. speciosus* (**Plate 3**). In the ethanolic extract from stem of *C. speciosus* was high of coumarin (25.632 ppm) compared to other phenolic compounds (**Plate 4**). Phloroglucinol was high (1.151 ppm) in the ethanolic extract from immature leaf of *C. speciosus* compared to other phenolic compounds (**Plate 5**).

Similarly, Orcinol was high (1.079 ppm) in the water extract from mature leaf of *C. speciosus*. Catechin was high (0.422 ppm) in the water extract from stem of *C. speciosus* compared with other phenolics. Phloroglucinol was high (6.107 ppm) in the water extract from immature leaf of *C. speciosus*. Both ethanol and acetone extract of mature leaf of *C. speciosus* contained high of ferulic acid. In immature leaf of *C. speciosus* had highest content of Phloroglucinol in both ethanol and water extract.

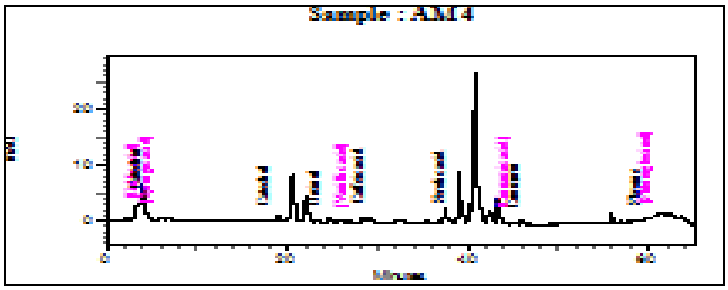

PLATE 2: HPLC CHROMATOGRAM OF PHENOLICS IN THE ACETONE

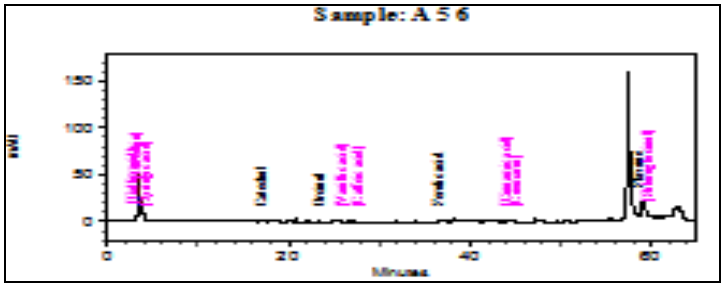

PLATE 3: HPLC CHROMATOGRAM OF DIFFERENT PHENOLICS IN THEEXTRACT FROM MATURE LEAF OF *COSTUS SPECIOSUS* ACETONE EXTRACT FROM STEM OF *COSTUS SPECIOSUS*

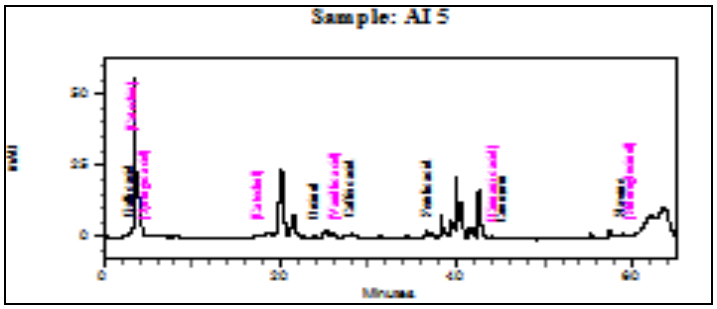

PLATE 4: HPLC CHROMATOGRAM OF PHENOLICS IN THE ACETONE

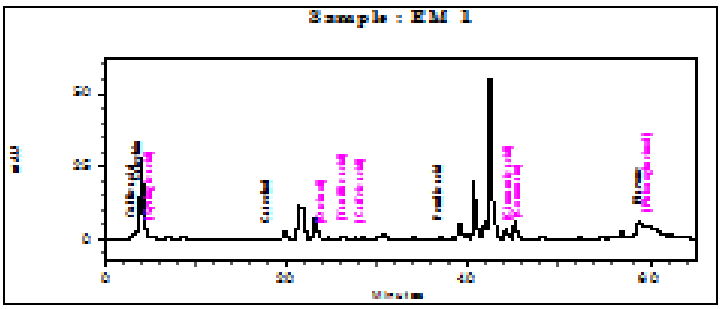

PLATE 5. HPLC CHROMATOGRAM OF PHENOLICS IN THE ETHANOLIC EXTRACT FROM IMMATURE LEAF OF *COSTUS SPECIOSUS* EXTRACT FROM MATURE LEAF OF *COSTUS SPECIOSUS*

HPLC currently represents the most popular and reliable technique for analysis of phenolic compounds. Various supports and mobile phases are available for the analysis of phenolics including anthocyanins, proanthocyanidins, hydrolysable tannins, flavonols, flavan-3-ols, flavanones, flavones, and phenolic acids in different plant extract and food samples [21-23].

The introduction of reversed-phase (RP) columns has considerably enhanced HPLC separation of different classes of phenolic compounds and RP C-18 columns are almost exclusively employed. It was found that column temperature may affect the separation of phenolics such as individual anthocyanin [24] and constant column temperature is recommended for reproducibility [25].

Acetonitrile and methanol are the most commonly used organic modifiers. In many cases, the mobile phase was acidified with a modifier such as acetic, formic, and phosphoric acid to minimize peak tailing.

Both isocratic and gradient elution are applied to separate phenolic compounds. The choice depends on the number and type of the analyte and the nature of the matrix. Several reviews have been published on application of HPLC methodologies for the analysis of phenolics [26].

The most often used method to measure the individual flavonoids and phenolic compounds is reversed-phase high performance liquid chromatography (RP-HPLC). HPLC allows a high resolution and a rapid and reproducible determination, even of trace amounts of these compounds.

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