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In Vitro and *In Silico* Screening For Potential Phytochemicals In Alcoholic Extract Of *Cheilocostus* Speciosus For Its Pharmacological Properties

Shalet Varghese, Baskaran. K*, Haseera. N, Nirmala Devi.N

Department of Biochemistry, Sree Narayana Guru College, Coimbatore, Tamilnadu, India.

***Correspondence Author*: Dr. K. Baskaran,** *MSC, M.Phil., Ph.D.
Assistant Professor, Department of Biochemistry,
Sree Narayana Gure College, Coimbatore-641105Tamil Nādu, India
E-mail: baskar.bio86@gmail.com Cell number: 91+8760302579

Abstract:
Cheilocostus speciosus is an erect perennial herb belonging to family Costaceae, an important medicinal plant widely used in several indigenous medicinal formulations. Presently, these plants could be collected from wild habitat only. Due to indiscriminate collection from natural habitat it has become endangered. In the present investigation, the phytochemical screening, Genomic DNA Isolation by Phenol Chloroform method and Phylogenetic analysis of the rhizome extracts of *Cheilocostus speciosus* were evaluated. Phytochemical screening indicated that, rhizomes are rich in a variety of primary metabolites such as Coumarin, Flavanoid, Terpenoid, Steroid, Tannin, carbohydrates, saponins, Cardiac glycosides, Anthraquinones. The solvents used were water, methanol, ethanol, ethyl acetate and chloroform. The present findings for *In vitro and In silco* screening for potential phytochemicals in alcoholic extract of *Cheilocostus speciosus* for its pharmacological properties suggested that their contents are responsible for significant antioxidant activity in all extracts.

Keywords: *Cheilocostus speciosus*, phytochemicals, pharmacological properties, rhizome, Genomic DNA.

INTRODUCTION

Cheilocostus speciosus is a perennial rhizomatous herb with erect or spreading stems commonly called as crepe ginger or spiral flag in English (Gupta, 2010). It is an erect plant up to 2.7 m high with tuberous root stalk, a sub-woody stem at the base flowers are larger, white, in thick, cone like terminal spikes, with bright red bracts. *Cheilocostus speciosus* is native to the Malay peninsula of the south-east Asia. In India, the plant naturalizes in sub-Himalayan tract of central India and parts of Western Ghats of Maharashtra, Karnataka and Kerala (Sarin *et al.*, 1974). It is known as Keukand, Keu, Kust (Hindi), Pakarmula (Gujrathi), Penva, Pushkarmula (Marathi), Kustha (Sanskrit) and Kostam (Tamil) (Anonymous, 2001). The rhizomes are bitter and show anthelmantic, astringent, depurative and expectorant properties (Chopra *et al.*, 2006; Anonymous, 2007; Bown, 2008; Nadkarni, 2009). It has antioxidant, antifungal, antituberculosis and oestrogenic activity. The rhizome extract is used as tonic and useful in reliving burning sensation, constipation, asthma, bronchitis, leprosy, anaemia and other skin alignments (Shivarajan and Balchandran, 1994). The rhizome of *Cheilocostus speciosus* has hepatoprotective properties (Bhuyan and Zaman, 2008). Rhizome paste is used for treating boils and used as contraceptive (Warrier *et al.*, 1993-1995). Rhizome possesses antifertility, anticholinesterace, anti-inflammatory, antipyretic and antihelminthic activities (Bhattacharya *et al.*, 1972;

Hussain *et al.*, 1992). Steroid saponins and sapogenins from *Cheilocostus speciosus* exhibited antifungal activity (Singh and Srivastava, 1992). In south-east Asia, it is used to treat boils, constipation, diarrhoea, dizziness, headache, ear, eye and nose pain, and used to stop vomiting. Japanese used the rhizome extract to control syphilis (Jain, 1991).

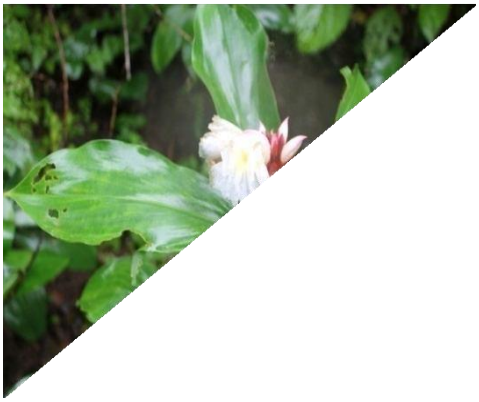

Fig. 1.*Cheilocostus speciosus* from kerala

Fig. 2. Rhizome of *Cheilocostus speciosus.*

Pharmacological studies showed that the rhizomes of *Cheilocostus speciosus* possess cardio tonic, hydrochloretic, diuretic and CNS depressant activity (Mahato *et al.*, 1980). The demand of nutraceuticals is increasing day-by-day, so herbs can be a better option for the replacement of synthetic antioxidant agents. Keeping the above facts in view, this study deals with the comparative antioxidant activity of multi-solvent extracts of the rhizome of *Cheilocostus speciosus* based on their vitamin, flavonoids and other natural antioxidants.

MATERIALS AND METHODS

Preparation of the plant extract

Fresh rhizomes of *Cheilocostus speciosus* (*Hellenia speciosa*) were sun dried for 7 days and finally autoclaved in an electric oven below 60 °C for 23 hours. The rhizomes were dried and crushed into fine powder. Extraction of dried rhizome powder was carried out in five different solvents with high, medium and low polarity. The solvents used were water, methanol, ethanol, ethyl acetate and chloroform. The sample was soaked in the above respective solvents in the ratio 1:5 and kept in shaker for 48 hours at 28°C. After 48 hours, the samples were filtered using Whatman no.1 filter paper. The solvents were then evaporated by using a hot water bath and the crude extracts obtained were stored in sterile glass bottles for further screening and analyses. Preparation of plant extracts with different polarity can be achieved using different solvents and extraction methods.

Preliminary Phytochemical Screening

Phytochemical identification was done to determine phytochemical content in samples such as Coumarin, Flavonoids, Terpenoid, Steroid, Tannin, carbohydrates, saponins, Cardiac glycosides, Anthraquinones in *Cheilocostus speciosus* rhizomes five extracts (Baskaran etal., 2023).

Genomic DNA Isolation by Phenol Chloroform method

The plant rhizomes were taken for the analysis (50mg wet weight). The tubes were incubated at 55°C for an hour and a half in a water bath (micro tubes were fixed with parafilm). 220 µl of PCI solution (saturated Phenol: Chloroform: Isoamyl alcohol; 25:24:1) was included and after that blended by using a micropestle. The tubes were centrifuged at 13,000 rpm for 15 minutes and the upper layer was taken in new micro tubes. Again 220 µl of PCI solution was included and centrifugation was rehashed for a moment time at 13,000 rpm for 15 min. The upper layer was taken in a new micro tube. A parallel measure of chloroform was added to the upper layer. The tubes were centrifuged at 13,000 rpm for 15 minutes and the fluid stage was gathered and exchanged to new micro tubes. A twofold volume of chilled absolute alcohol was included and left overnight in the -20°C fridge (kept for 1-2 hours). The tubes were centrifuged at 13,000 rpm for 20 minutes to get a DNA pellet, and the supernatant was disposed of. 300 µl of super cold 70% alcohol was included for washing the pellet taken after centrifugation at 13,000 rpm for 10 minutes. The ethanol was painstakingly poured off and was permitted to dry for 30-45 minutes at 37°C (in an incubator). 50 µl of hydration buffer

(1x MilliQ TE) was added and permitted to rehydrate at room temperature for 10 minutes and afterwards put away in a 4°C cooler. Electrophoresis was performed on a 0.8% agarose gel for checking the quality and quantity of DNA. Spectrophotometric analysis was also done for analysing the same.

Phylogenetic analysis

Phylogeny is the study of the evolutionary relationships among different species or groups of organisms. It is often represented in the form of a tree-like diagram, known as a phylogenetic tree or evolutionary tree, which shows the inferred evolutionary history of the different taxa being studied. Phylogenetic trees are constructed based on the comparison of homologous traits, such as DNA or protein sequences, morphological characteristics, or behavioural traits, among the different organisms. The idea is that organisms that share more similarities in their traits are more closely related and have a more recent common ancestor than those that are less similar.

Phylogenetic has numerous applications in fields such as evolutionary biology, ecology, conservation, and biogeography. For example, it can be used to infer the origin and diversification of different groups of organisms, to reconstruct the history of life on Earth, to identify key events in the evolution of particular traits, and to inform conservation efforts by identifying groups of organisms that are most at risk of extinction. It can also help in understanding the emergence of diseases by mapping out the genetic relationship of different strains. Building a phylogenetic tree requires four distinct steps: (Step 1) identify and acquire a set of homologous DNA or protein sequences, (Step 2) align those sequences, (Step 3) estimate a tree from the aligned sequences, and (Step 4) present that tree in such a way as to clearly convey the relevant information to others. The most reliable way to identify sequences that are homologous to the sequence of interest is to do a Basic Local Alignment Search Tool (BLAST) search (Altschul et al. 1997) using the sequence of interest as a query. There are several widely used methods for estimating phylogenetic trees (Neighbor Joining, UPGMA Maximum Parsimony, Bayesian Inference, and Maximum Likelihood [ML]), In this study, MEGA 11 was used to analyse evolutionary relationship. MEGA （Molecular Evolutionary Genetics Analysis）is an integrated tool for automatic and manual sequence alignment, inferring Phylogenetic trees, mining web-based databases, estimating rates of molecular evolution, and testing evolutionary hypotheses.

Statistical analysis

All the assays were carried out in triplicate. Experimental results are expressed as mean ± standard deviation. The results were analyzed using one-way analysis of variance and the group means were compared using Duncan's multiple range tests using SPSS version 16.

RESULTS AND DISCUSSION

Preliminary phytochemical analysis (secondary metabolites)

The plant extract was obtained in five polar to non- polar fractions and were subjected to phytochemical analysis which yielded following result.

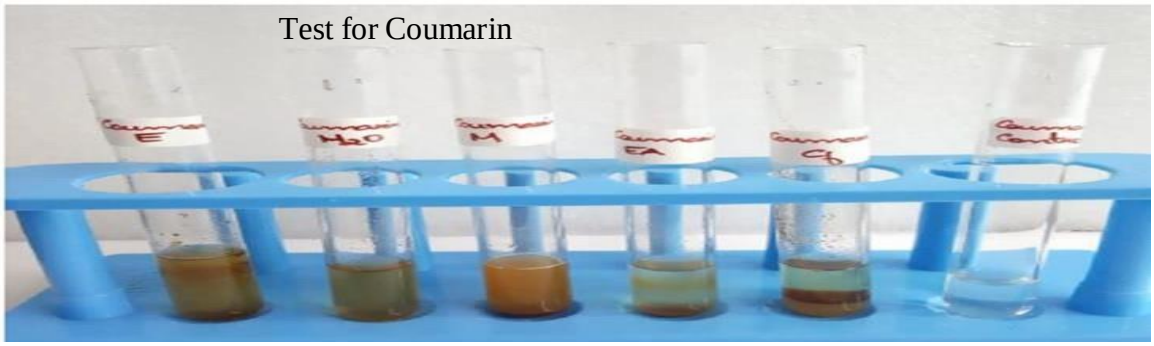

Fig.3. *Chielocostus speciosus* five extract test for Coumarin

Test for Coumarin: *Cheilocostus speciosus*, has been reported to contain Coumarin, among other phytochemicals. Coumarin isolated from *Cheilocostus speciosus* has exhibited various biological activities, including antioxidant, anti-inflammatory, and antimicrobial properties. Additionally, some studies have reported the potential anticancer effects of Coumarin extracted from this plant. However, further research is

needed to explore the full pharmacological potential of coumarins from *Cheilocostus speciosus* and their possible applications in medicine and related industries. Coumarins can be found in many natural sources such as Tonka beans, cinnamon, sweet woodruff, and licorice, and they are also synthesized for various applications in industries such as perfumes, flavourings, and cosmetics. In this study, yellow coloured fluorescence was observed in the methanol extract of the plant extract.

Test for flavonoids: Some common flavonoids found in plants include quercetin, kaempferol, rutin, apigenin, luteolin, and hesperidin. Flavonoids are synthesized in plants through the shikimate and phenylpropanoid pathways, and they serve a variety of functions, including UV protection, pigmentation, pollinator attraction, and defence against herbivores and pathogens. Flavonoids have been studied for their potential health benefits, as they have been shown to possess antioxidant, anti-inflammatory, and anti-carcinogenic properties. They may also have cardio protective effects, and may help reduce the risk of chronic diseases such as diabetes, cardiovascular disease, and cancer. Flavonoids are commonly consumed in the diet, as they are present in a variety of plant-based foods, such as fruits, vegetables, nuts, and legumes. However, the bioavailability of flavonoids varies depending on the food source and the individual consuming them, and more research is needed to fully understand the potential health benefits of flavonoids. Flavonoids found in *Cheilocostus speciosus* include quercetin, kaempferol, and rutin. In this study, green colouration was found in water containing fraction and orange colouration in methanol containing fraction which indicates the presence of flavonoids.

Fig.4. *Cheilocostus speciosus* five extract test for flavonoids

Test for Terpenoid: Terpenoids are responsible for the characteristic odours and flavours of many plants, such as the smell of pine trees, the taste of cinnamon, and the scent of lavender. They are used in the food and beverage industry as flavourings and fragrances and in the pharmaceutical industry as active ingredients in drugs. Some of the terpenoids that have been identified in *Cheilocostus speciosus* include β-caryophyllene, α-pinene, β-pinene, camphene, limonene, and linalool. These terpenoids have been found to possess various biological activities, such as anti-inflammatory, antimicrobial, and antioxidant properties. In this study, red-brown colour at the interface was observed in the case of ethanol, methanol, ethyl acetate and chloroform extract indicating terpenoid.

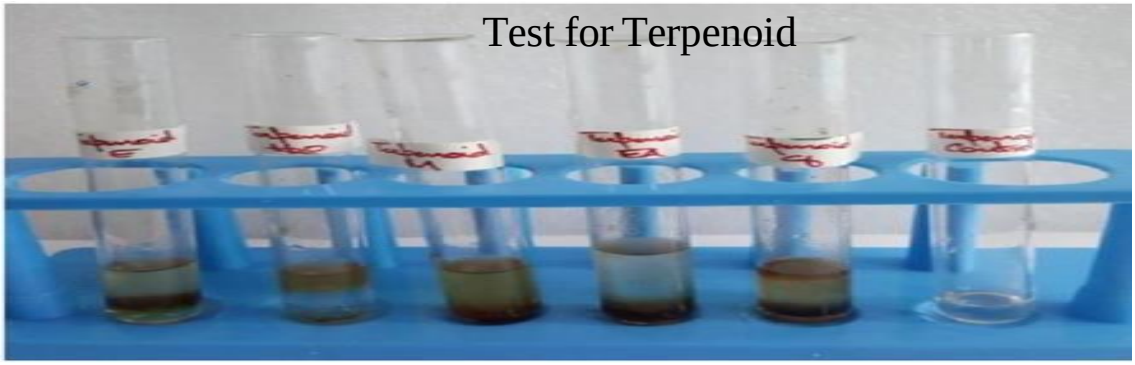

Fig.5. *Cheilocostus speciosus* five extract test for Terpenoids

Test for steroid: Some of the most common phytosterols found in plants include β-sitosterol, campesterol, and stigmasterol. These compounds have been shown to have various health benefits, including the ability to reduce cholesterol absorption and improve cardiovascular health. They are also believed to have anti-

inflammatory and anticancer properties. Phytosterols are widely used as food additives and supplements due to their potential health benefits. They are often added to margarine, spreads, and other food products to improve their nutritional profile and provide a cholesterol-lowering effect. However, it is important to note that excessive intake of phytosterols may have adverse effects on health, including interference with the absorption of fat-soluble vitamins and potential disruption of normal cholesterol metabolism. In this study, all the fractions tested did not show any presence of the steroid.

Fig.6. *Cheilocostus speciosus* five extract test for steroid

Test for tannin: Tannins are produced by the plant to protect it from predators and to help prevent decay. Tannins are widely distributed in nature and are found in many food and beverage products, such as wine, tea, coffee, and chocolate. In traditional medicine, *Cheilocostus speciosus* has been used for the treatment of various ailments, including inflammation, pain, and skin infections. However, in the present study the test conducted shows that tannins were not present in any of the 5 fractions tested.

Fig.7. Cheilocostus *speciosus* five extract test for tannin

Test for carbohydrate: Plants store carbohydrates in various forms, such as starch, cellulose, and hemicellulose. Starch is the primary storage carbohydrate in plants, and it is found in various plant organs such as roots, tubers, and seeds. Cellulose and hemicellulose are structural carbohydrates found in the cell walls of plants, providing strength and rigidity to the plant structure. Carbohydrates in plants are not only important for the plants own metabolism but also serve as a crucial source of nutrition for other organisms, such as herbivores and humans. Carbohydrates are an essential component of the human diet and provide energy for various physiological processes. The study conducted to identify carbohydrates showed the presence in the entire fraction tested.

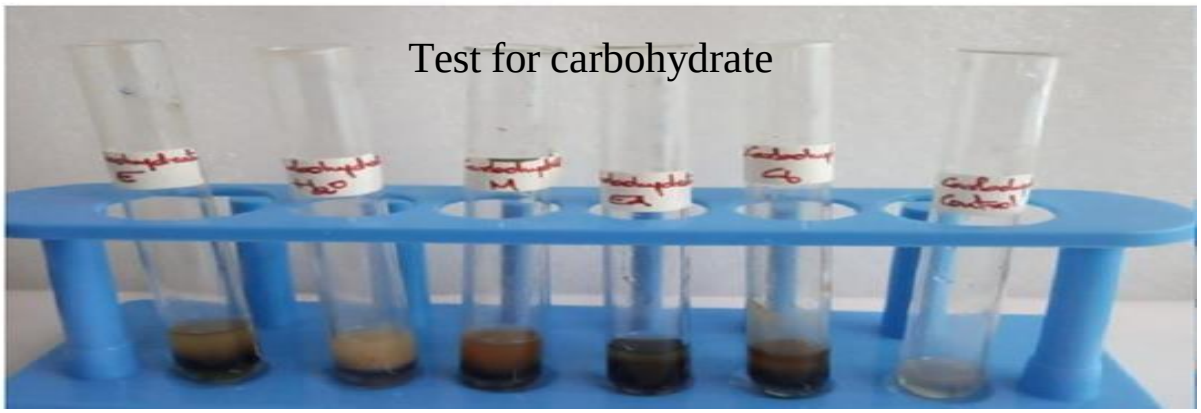

Fig.8. *Cheilocostus speciosus* five extract test for carbohydrate

Test for saponins: In the pharmaceutical industry, saponins are used as active ingredients in drugs for their therapeutic properties. For example, the saponins in the plant extract of the yucca plant have been used to treat arthritis, migraines, and high blood pressure. Additionally, saponins have been found to have antifungal, antibacterial, and antiviral properties, making them useful in developing treatments for infectious diseases. *Cheilocostus speciosus* is reported to contain various bioactive compounds, including saponins. The present study indicated that saponins are present in this plant as indicated by the frothy appearance in the methanol extract.

Fig.9. *Cheilocostus speciosus* five extract test for saponins

Test for glycosides: A study published in the Journal of Pharmacognosy and Phytochemistry in 2016 analyzed the phytochemical composition of *Cheilocostus speciosus* and reported the presence of cardiac glycosides in the plant extract. However, further studies are needed to confirm the presence and quantity of cardiac glycosides in this plant species. The present study indicated the presence of cardiac glycoside in all the extracts except that of water.

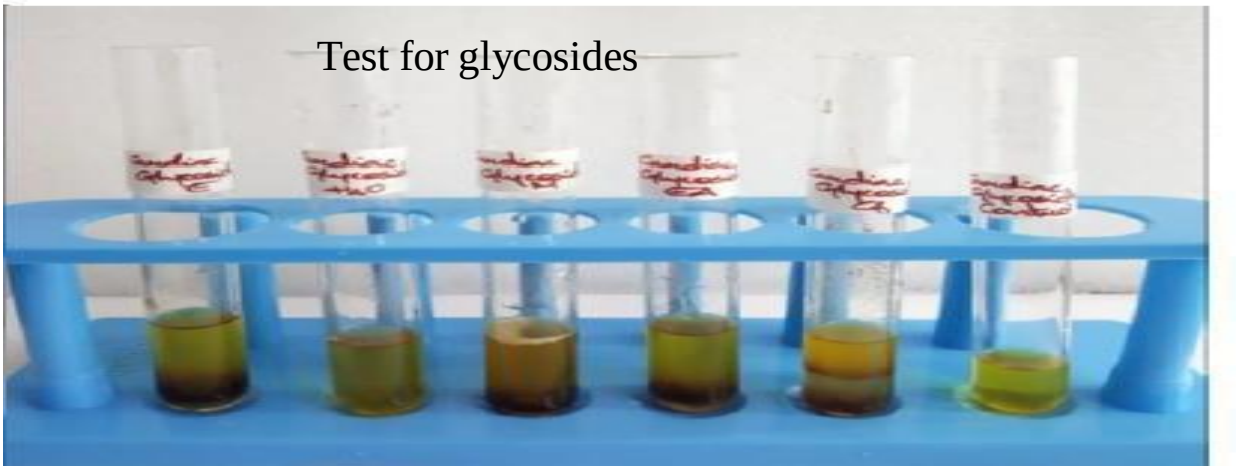

Fig.10. *Cheilocostus speciosus* five extract test for glycosides

Test for Anthraquinones: Anthraquinone is known for its medicinal properties and has been used traditionally as a laxative, anti-inflammatory, and antibacterial agent. It is also used as a dye in the textile industry, where it is known for its bright and long-lasting colors. Anthraquinones are commonly found in plants such as aloe vera, senna, rhubarb, and cascara sagrada. Anthraquinone derivatives, such as emodin and aloe emodin, have been extensively studied for their pharmacological activities. These compounds have been shown to have anti-inflammatory, anti-cancer, anti-viral, and anti-bacterial properties. They are also known to modulate various signalling pathways in the body, including those involved in apoptosis, inflammation, and oxidative stress. The present study conducted to identify the presence of anthroquinone in the plant extracts yielded negative.

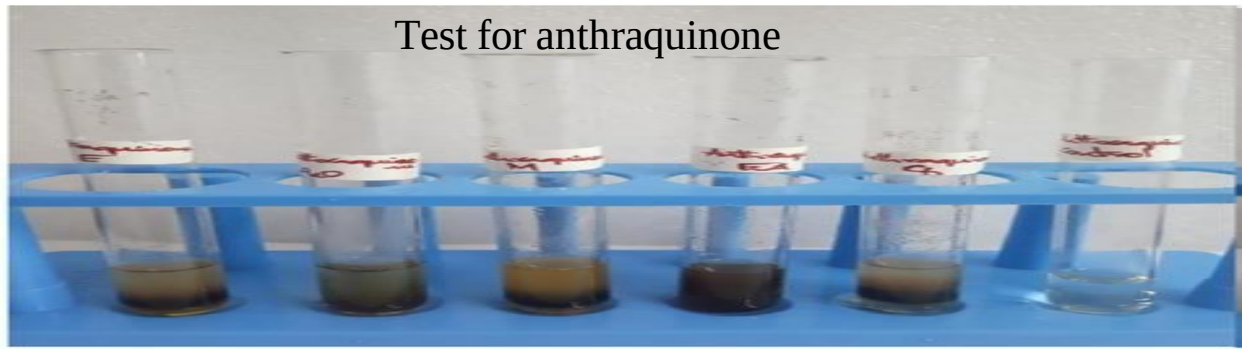

***Fig.11. Cheilocostus speciosus* five extract test for anthraquinone**

Test for alkaloid: One of the major alkaloids found in *Cheilocostus* is costunolide, which has been reported to have various pharmacological activities including anti-inflammatory, antioxidant, and anticancer effects. Costunolide has been shown to inhibit the growth of various cancer cells such as breast cancer, prostate cancer, and lung cancer cells. Another alkaloid found in *Cheilocostus speciosus* is dehydrocostus lactone, which has been reported to have anti-inflammatory and analgesic effects. It has been shown to inhibit the production of inflammatory cytokines and decrease the expression of pro-inflammatory enzymes in cells. Other alkaloids reported in Cheilocostus speciosus include 6-alpha-hydroxymusizin, costunolide diepoxide, costus acid, and iso-costic acid. These alkaloids have also been reported to have various pharmacological properties such as anti-inflammatory, anti-bacterial, and anti-cancer effects. The study conducted to identify the presence of alkaloids showed positive result in the ethanol, ethyl acetate and chloroform fraction.

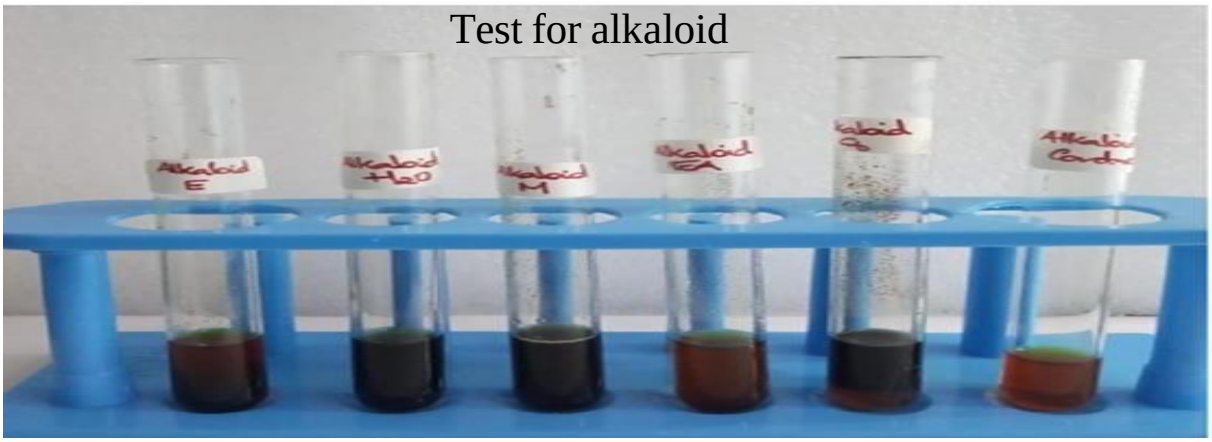

Fig.11. Cheilocostus speciosus five extract test for alkaloid

Table .1. Phytochemical screening of *Cheilocostus speciosus* rhizomes five extracts

Sl. No	Test	Ethanol	Water	Methanol	Ethyl acetate	Chloroform
1	Coumarin	-	-	-	-	-
2	Flavanoid	-	+	+	-	-
3	Terpenoid	+	-	+	+	+
4	Steroid	-	-	-	-	-
5	Tannin	-	-	-	-	-
6	Carbohydrate	+	+	+	+	+
7	Saponin	-	-	+	-	-
8	Cardiac glycoside	+	-	+	+	+
9	Anthraquinones	-	-	-	-	-
10	Alkaloid	+	-	-	+	+

Molecular taxonomic identification of plant

Molecular identity of the plant was confirmed by PCR of rcbL region followed by Sanger's sequencing, NCBI BLAST and phylogeny.

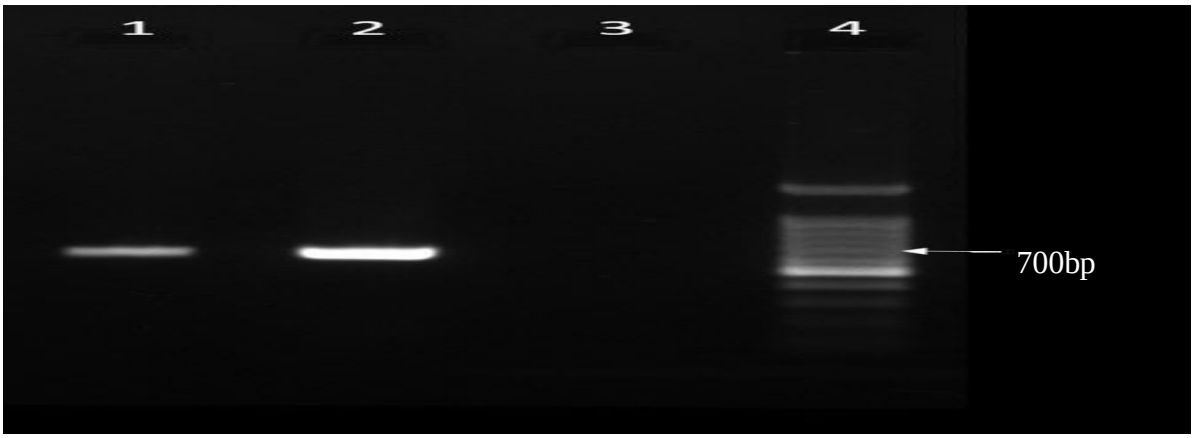

Fig.13. Gel electrophoresis image of PCR amplification

Lane 1: Positive Control, Lane 2: Test, Lane 3: Negative control

BLAST result for the sequence: The sequence (***Hellenia speciosa*** strain ***Cheilocostus speciosus***) obtained was subjected to BLAST analysis using NCBI-BLAST tool. This is carried out for sequence similarity search.

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Hellenia speciosa isolate L-67.1091 plastid, partial genome	Hellenia speciosa	2636	2636	100%	0.0	99.86%	130196	MH603405.1
Tapeinochilos ananassae voucher Zomlefer et al 2303 (FTG, GA, NY) isolate 83458B plastid, partial genome	Tapeinochilos an...	2551	2551	100%	0.0	98.75%	130368	MH603446.1
Costus tonkinensis isolate Yunnan chloroplast, complete genome	Costus tonkinensis	2532	2532	100%	0.0	98.60%	166360	OP712650.1
Costaceae sp. SJZ-2021b voucher IBSC:20090406 chloroplast, partial genome	Parahellenia yun...	2532	2532	100%	0.0	98.60%	166652	OL688998.1
Hellenia speciosa isolate Guangdong chloroplast, complete genome	Hellenia speciosa	2490	2490	100%	0.0	97.80%	167174	OP712649.1
Hellenia viridis voucher IBSC:20082501 chloroplast, partial genome	Hellenia viridis	2490	2490	100%	0.0	97.80%	166934	OL688999.1
Hellenia speciosa voucher IBSC:005 chloroplast, partial genome	Hellenia speciosa	2490	2490	100%	0.0	97.80%	167011	OL688995.1
Hellenia speciosa chloroplast, complete genome	Hellenia speciosa	2490	2490	100%	0.0	97.80%	167626	NC_066960.1
Hellenia speciosa chloroplast, complete genome	Hellenia speciosa	2490	2490	100%	0.0	97.80%	167626	ON598392.1
Hellenia speciosa chloroplast, complete genome	Hellenia speciosa	2490	2490	100%	0.0	97.80%	167158	OK641589.1
Costus sp. DML-2022a chloroplast, complete genome	Costus sp. DML-...	2484	2484	100%	0.0	97.73%	167185	OP712652.1
Hellenia deliniana voucher IBSC:20091003 chloroplast, partial genome	Hellenia deliniana	2484	2484	100%	0.0	97.73%	168082	OL689000.1
Hellenia lacera chloroplast, complete genome	Hellenia lacera	2484	2484	100%	0.0	97.73%	168053	NC_066959.1
Hellenia lacera chloroplast, complete genome	Hellenia lacera	2484	2484	100%	0.0	97.73%	168053	ON598391.1
Hellenia oblonga voucher IBSC:20082507 chloroplast, partial genome	Hellenia oblonga	2447	2447	100%	0.0	97.20%	166850	OL688997.1

Based on BLAST analysis, sample (*Hellenia speciosa* strain *Cheilocostus speciosus* shows 99.86% with *Hellenia speciosa* (Accession no. MH603405.1) with a query coverage of 100%, which implies that the sample may be *Hellenia speciosa*.

Phylogenetic analysis

Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-2586.24) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 7 nucleotide sequences. Codon

positions included were 1st+2nd+3rd+Noncoding. There were a total of 1431 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

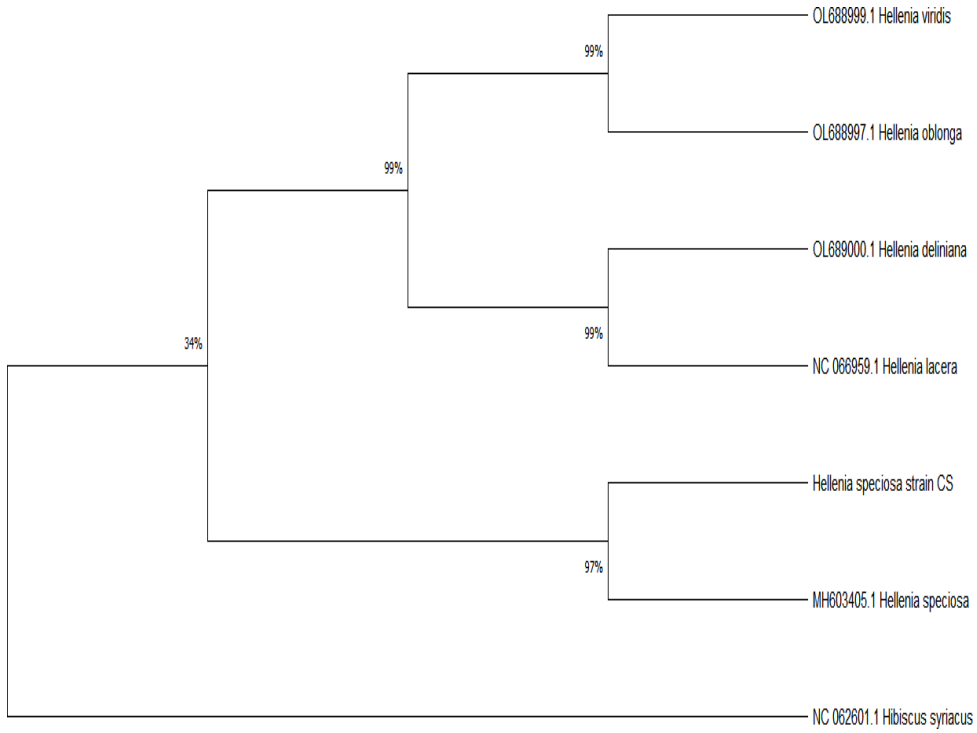

Fig.14. Phylogenetic Grouping of (*Hellenia speciosa* strain *Cheilocostus speciosus*)

CONCLUSIONS

The present study shows that the plant *Cheilocostus speciosus* rhizomes five extracts has phytochemicals like Coumarin, Flavonoids, Terpenoid, Steroid, Tannin, carbohydrates, saponins, Cardiac glycosides, Anthraquinones. Each phytochemical has its own medicinal property. The result obtained in this study showed that *Cheilocostus speciosus* extract has phytochemical screening, Molecular taxonomic identification of plant and Phylogenetic analysis with *Cheilocostus speciosus* extract.

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