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STUDIES ON THE ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF BIOACTIVE COMPOUND FROM *COSTUS IGNEUS* LEAF EXTRACTS

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Abstract

Costus igneus, commonly known as the insulin plant, is a Spiral Flag member of the family *Costaceae* and a newly introduced plant in India from South and Central America. It is a perennial, upright, spreading plant, reaching about two feet tall, with spirally arranged leaves and attractive flowers. It usually grows as an ornamental plant in southern India, and its leaves are used as a dietary supplement in the treatment of diabetes mellitus. It has been traditionally reported for its anti-diabetic, anti-oxidant, anti-inflammatory, anti-proliferative, anti-urolithiasis, hypolipidemic, neuroprotective, and anti-microbial activity. In this study, the phytoprofiling of *Costus igneus* leaf extracts showed better extraction with ethanol solvent. The chromatographic and spectral output showed the presence of bioactive compounds and Cyclotrisiloxane, hexamethyl-(cyclic hydrocarbon) possessed and the anti-microbial and anti-oxidant were purified and definite.

Keywords: *Costus igneus*, anti-diabetic, anti-oxidant, anti-inflammatory, anti-proliferative, anti-urolithiasis, hypolipidemic, neuroprotective ,anti-microbial activity, phytoprofilling, Gas chromatography-Mass spectroscopy (GC-MS), Cyclotrisiloxane, hexamethyl-, Nuclear magnetic resonance (NMR).

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Introduction

Costus igneus, commonly known as Fiery Costus, Step Ladder, Spiral Flag, or Insulin Plant, is native to South and Central America. This is a recent introduction to India from America as an herbal cure for diabetes and is hence commonly called the "Insulin plant" (Jose B, Reddy LJ., 2010). It is widely grown in gardens as an ornamental plant in South India and also runs wild in many places (Benny M., 2004). *Costus igneus* has been traditionally reported for its anti-diabetic, anti-oxidant, anti-inflammatory, anti-proliferative, anti-urolithiasis, hypolipidemic, neuroprotective, and anti-microbial activity. This review is all about anatomical and morphological investigations of *Costus igneus*, including its medicinal use. Secondary metabolites of this plant, such as b-sitosterol, corosolic acid, diosgenin, quercetin, catechine, and oleic acid, show mainly anti-diabetic activity. At present, *Costus igneus* has characteristic morphological, anatomical, and proximate features that could be used to differentiate it from other members of the *Costaceae* family (Shinde *et al*., 2022). It is used in India to control diabetes, and it is known that diabetic people eat one leaf daily to keep their blood glucose low (Devi VD, Urooj A., 2008). The leaves of *C. igneus* were one of the plants known to be effectively used for diabetes treatment by the tribal people of Namakkal district, Tamilnadu (Elavarasi S, Saravanan K., 2012).

Materials and Methodology

Sample Collection

The study plants were collected from around Pechiparai and Citraru dams located in Kanyakumari district. The collected plants were allowed to shed dry for 7 days, followed by the grinding of leaf samples.

Solvent Extraction by Percolation

About 5 grams of fine ground leaf powder was subjected to the best compound extraction system by employing 50 mL of different polar (Aqueous, Ethanol, Acetone, and DMSO) and non-polar (Diethyl ether and Hexane) solvents, respectively.

Screening of Phytochemicals

Flavonoids (shinoda test)

Detection of flavonoids was done by using the Kokate, 1994 method. To 2 ml of each leaf extract, a few drops of concentrated hydrochloric acid were added. Also 0.5 grams of magnesium turnings were added to the solution. The formation of a pink colour indicated the presence of flavonoids.

Phenol (Ferric chloride test)

The Mukherjee (2002) method was followed to analyse the phenolic content of study extracts of *Costus igneus* leaf samples. The extracts were diluted to 5 mL with distilled water. To that, a few drops of neutral 5% ferric chloride solution were added. A dark green colour formation indicates the presence of phenolic compounds in test extracts.

Triterpenoids

The triterpenoid was detected with the protocol described by Horbone (1984). To the test of different study extracts, 2 mL of chloroform was added with 3 mL of concentrated sulphuric acid at the side of the test tube. An interface with a reddish brown colour formation is indicated by the presence of triterpenoids.

Soxhlet Extraction

About 10 grams of *Costus igneus* leaf powdered was packed in clean sterile extraction bag and loaded in soxhlet apparatus with 100 mL of ethanol solvents. The crude ethanolic extraction was achieved by heating the soxhlet at 54 ± 1 °C for 24 hrs. After extraction, the collected crude sample was refrigerated for further experiments.

Quantitative Estimation of Terpenoids

To the 1.5 mL Chloroform, add 200µl of ethanolic extract of *Costus igneus* in test

tube. A standard (Linalool solution) were prepared by diluting with 1.5 ml Chloroform with the concentration of 100mg/200µl to 1mg/200µl (12.965 µM - 1.296 µM). Vortex the sample mixture thoroughly and stand for 3 minutes. Add 100µl Concentrate Sulfuric acid (H_2SO_4) to each sample containing tubes. Then the assay tubes were kept for incubation at room temperature for 1.5 hrs - 2hrs in dark. At the end of incubation time a reddish brown precipitation will be formed in each assay tubes. Discarded the supernatant carefully without disturbing the precipitation. Add 1.5 ml of 95% (Vol/Vol) Methanol & vortex thoroughly until all the precipitation dissolve in Methanol completely. Transfer the sample from assay tube to Colorimetric cuvette [95% (Vol/Vol) Methanol will be used as blank] to read the absorbance at 538 nm (Bhalla, K.N. (2003), Bifulco, M (2005) and Finar, I.L (1964).

Compound Identification

Thin Layer Chromatography

The mobile phase was prepared by dissolving the ethyl acetate. Formic acid: Glacial acetic acid: Water in the ratio of 10:1.1:1.1:2.6 for flavonoids, Tolune: Acetone: Formic acid (4.5:4.5:1) for phenols, and Tolune: ethyl acetate (93:7) for triterpenoids. And about 10l of ethanolic extracts of the respective samples (*Costus igneus*) were dropped on the TLC sheet 2 cm above the bottom. Allow the chromatographic chamber for the separation of compounds as individual bands. Then a chromatogram was developed by sparing with the following visualising agents: 1% ethanolic aluminium chloride used for flavonoids and Folin Ciocalteu or Methanol + Conc. Sulfuric acid (95ml + 5ml for phenols. followed by observing the TLC band under UV at 325 nm. After the chromatogram was developed, the Rf (Retention Factor) was calculated by using the formula,

$$Rf = \frac{Distance\ travelled\ by\ Solute}{Distamce\ travelled\ by\ Solvent}$$

Gas chromatography Mass spectrophotometer

The crude ethanolic extract was subjected for GC-MS analysis in Agilent Technologies: GC-MS (GC System-7820A) with the analysis parameters of Over Temp -100 ^oC-270 ^{o} C (10 ^oC/min), Flow rate -1.2 and helium gas was setas mobile phase.

Isolation and Identification of Bioactive Compound

The ethanolic extract was further subjected to compound purification using silica gel column chromatography with the mobile phase of ethyl acetate and hexane (1:1). The ethanolic extract was loaded on top of the column at about 4-5 mL followed by solvent elution. The collected 6 fractions were analyzed for the presence of chromatographic bands in TLC by calculating the Rf value. Further, the chromatographic fraction-1 was studied for the presence of Cyclotrisiloxane, a hexamethyl-(cyclic hydrocarbon) compound. Later, the compound was structurally confirmed by Carbon (C^{13}) and Proton (H^1) NMR spectral analysis with the Bruker Avance 400 MHz FT-NMR Spectrometer.

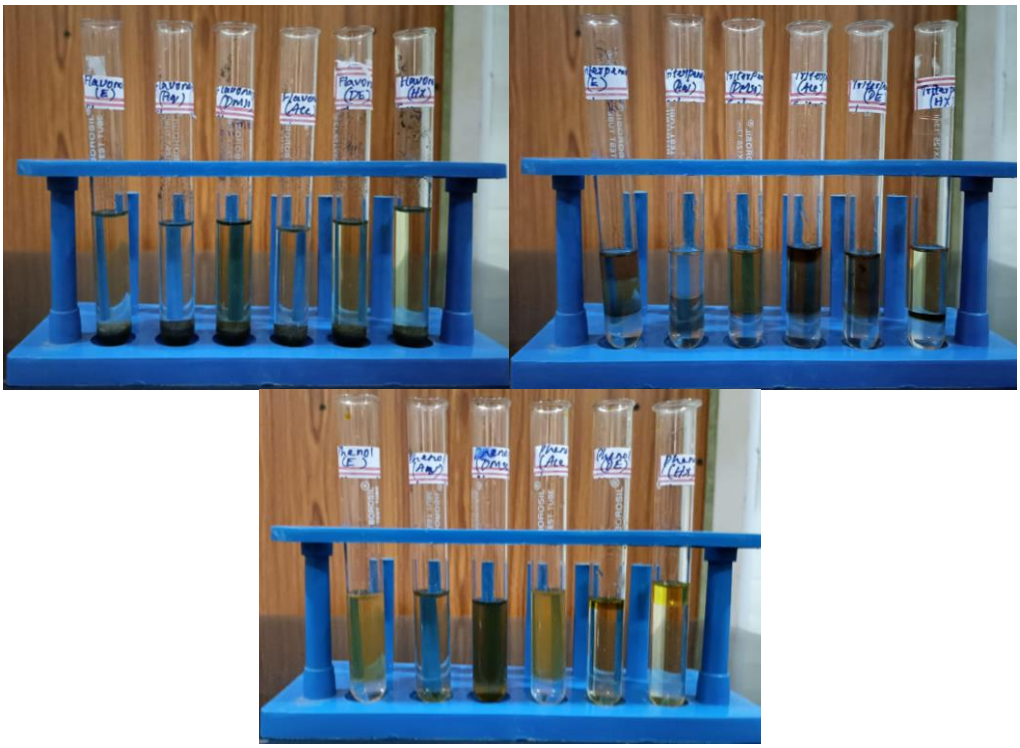
Results

Screening of Phytochemicals

Costus igneus leaf was extracted by different polar and non-polar solvents. All the extracted solvents were subjected to qualitative determination of phytochemicals. The results showed the presence of triterpenoids in polar (Ethanol, acetone and DMSO) and non-polar (Diethyl ether and Hexane) solvents except aqueous solvent while Phenol alone was detected in DMSO extract (**Table: 1 and Figure: 1**).

Table: 1. Qualitative Phytochemical analysis of different solvent extracts of *C. igneus*

Test name	*Costus igneus*		
	Phenol	**Flavonoids**	**Triterpenoids**
Aqueous	-	-	**Absent**
Ethanol	-	-	+
DMSO	+	-	+
Acetone	-	-	+
Diethyl ether	-	-	+
Hexane	-	-	+



Figures: 1. Qualitative Phytochemical analysis of different leaf extracts of *C. igneus*

Estimation of Triterpenoids

The concentration of triterpenoids in both polar and non-polar extracts of *Costus igneus* leaf was estimated, and the data revealed that the highest concentration of triterpenoids was measured in ethanolic extract at approximately 91.52 percent. Other hand, DMSO yielded 11.47% and acetone showed 5.59%. Non-polar solvents such as diethyl ether and hexane revealed 12.53% and 1.55% of triterpenoids, respectively. (**Table: 2**).

Table: 2. Quantitative analysis of Triterpenoids from different solvent extracts of *C. igneus*

Solvent Extract	OD I	OD II	OD III	Mean OD	Percentage of Terpenoids
Aqueous	-	-	-		-
Ethanol	0.854	0.854	0.856	0.09	91.52
DMSO	0.893	0.892	0.892	0.89	11.47
Acetone	0.950	0.953	0.952	0.95	5.59
Diethyl ether	0.882	0.881	0.882	0.88	12.53
Hexane	0.990	0.992	0.995	0.99	1.55

Thin Layer Chromatography

According to the Phytochemical screening, ethanolic extract of *Costus igneus* revealed the finest extraction system for triterpenoids. Further, this has been confirmed with thin layer chromatography with the observation of a light orange band with an Rf value of 0.79, indicating the presence of triterpenoids. (**Table: 3 and Figure: 2**).

Table: 3 Retention factor values of ethanolic extracts of *C. igneus*

S. No	Obtained Rf Value	Band Intensity and Colour		Detected Compound
		Visible	UV	
1.	-	Nil	Nil	Flavonoid
2.	0.8	Dark blue	Dark blue	Phenol
3.	0.79	Nil	Light Orange	Triterpenoids

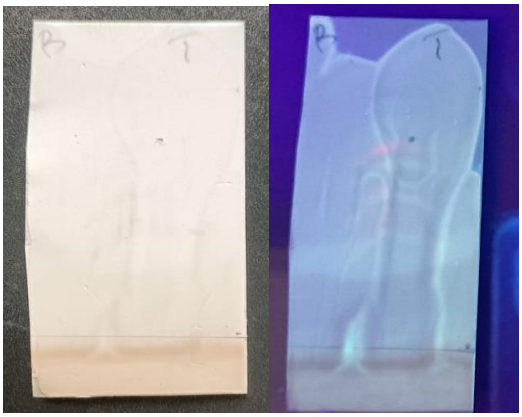
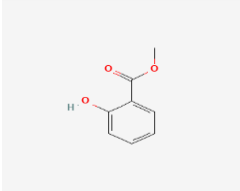
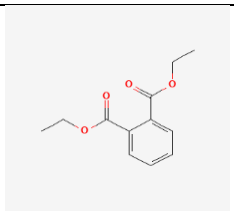
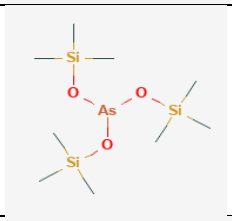
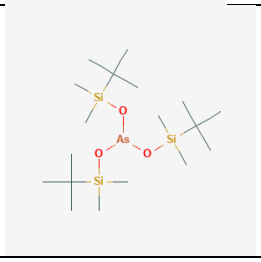


Figure: 2. Detection of Triterpenoids in ethanolic extract of *Costus igneus* TLC by UV light

Identification of Phytocompounds using GC-MS

The crude leaf ethanolic extract *Costus igneus* was employed for the identification of phytocompounds and the results revealed the presence of Methyl salicylate (Aromatic carboxylic acid ester) at the retention time of 3.619 mins, Diethyl Phthalate (Aromatic carboxylic acid ester) at 7.893 mins, Arsenous acid, tris(trimethylsilyl) ester, Tris(tert-butyldimethylsilyloxy)arsane and Indole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl- at 17.007 mins. Cyclotrisiloxane, hexamethyl- (Cyclic hydrocarbon) and 3, 3-Diisopropoxy-1,1,1,5,5,5-hexamethyltrisiloxane detected at 17.301 mins. The compound Benzene, 2-[(tert-butyldimethylsilyl) oxy]-1-isopropyl-4-methyl was found to be at 19.664 mins of retention time. While, Thieno[2,3-c]furan-3-carbonitrile, 2-amino-4,6-dihydro-4,4,6,6-tetramethyl- and N-Methyl-1-adamantaneacetamide were identified with the peaks at 19.712 mins (**Table: 4 and Figure: 3**).

Table: 4. Identification compounds in ethanolic extract of *Costus igneus* by GC-MS

S. No	Chemical Compounds	Retention Time (min)	CAS Number	Molecular Formula	Molecular Structure	Biological Activity
1	Methyl salicylate (Aromatic carboxylic acid ester)	3.619	000119-36-8	$C_8H_8O_3$		Anti-Microbial, anti-oxidant and anti-cancer (Essien, E. E.,2015) Anti-inflammatory (Li J., 2016)
2	Diethyl Phthalate (Aromatic carboxylic acid ester)	7.893	000084-66-2	$C_{12}H_{14}O_4$		antimicrobial, acetylcholinesterase, and neurotoxic (Velanganni J., 2011)
3	Arsenous acid, tris (trimethylsilyl) ester	17.007	05542 9-29-3	$C_9H_{27}AsO_3Si_3$		No activity
4	Tris(tert-butyldimethylsilyloxy)arsane	17.007	10003 66-57-5	$C_{18}H_{45}AsO_3Si_3$		No activity

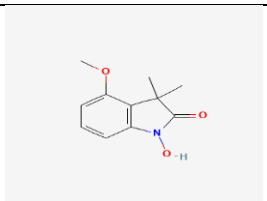
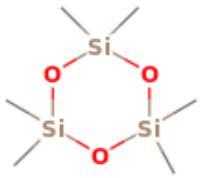
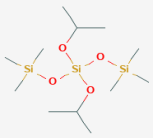
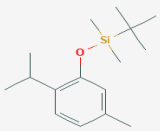
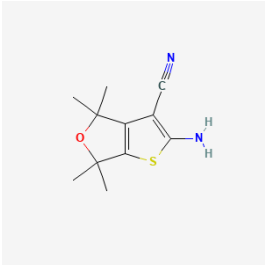
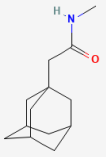
5	Indole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl-	17.007	100012 9-52-1	$C_{11}H_{13}NO_3$		Antimicrobial
6	Cyclotrisiloxane, hexamethyl- (Cyclic hydrocarbon)	17.301	00054 1-05-9	$C_6H_{18}O_3Si_3$		Anti-microbial and Anti-oxidant (S.R, Anjukrishna. *et al*., 2015)
7	3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyltrisiloxane	17.301	01808 2-56-9	$C_{12}H_{32}O_4Si_3$		No activity
8	Benzene, 2-[(tert-butyldimethylsilyl)oxy]-1-isopropyl-4-methyl-	19.664	33045 5-64-6	$C_{16}H_{28}OSi$		No activity
9	Thieno[2,3-c]furan-3-carbonitrile, 2-amino-4,6-dihydro-4,4,6,6-tetramethyl-	19.712	44741 2-24-0	$C_{11}H_{14}N_2OS$		Analgesic, Antianginal, Analgesic, non-opioid, Antihypertensive, Antiarthritic, Dementia treatment, Neurotransmitter uptake inhibitor (Brintha S. *et al*., 2017)
10	N-Methyl-1-adamantaneacetamide	19.712	03189 7- 93-5	$C_{13}H_{21}NO$		Antimicrobial (Rateb, H.S. *et al*., 2016) and COX-2 Inhibitor (Kakarla, Lavanya. *et al*., 2014)

Figure: 3 Chromatographic Peaks Chemical Compounds of Sample *Costus igneus* ethanolic extract by GC-MS

Compound isolation and Identification

Chromatographic Approaches

The ethanolic extract of *Costus igneus* leaf was subjected to column purification with ethyl acetate and hexane (1:1) as mobile phases. We collected around 6 elutents and further analysed the banding patterns on TLC. The TLC sheet was run with the sample mobile phase used as before, and the sheet was developed for the presence of bands. Fraction-1, 3, and 4 revealed the presence of triterpenoid bands. (**Table: 5 and Figure: 4**).

Table: 5. Retention factor values column fractions of *C. igneus*

S. No	Fractions	Spots	Triterpenoids	Observation	
				Vis	UV
1.	**F1**	**S1**	0.51	-	Light purple
		S2	0.89	-	Dark pink
2.	**F2**	**S1**	-	-	-
		S2	-	-	-
3.	**F3**	**S1**	0.47	-	Dark pink
		S2	0.93	-	Dark pink
4.	**F4**	**S1**	0.89	-	Light purple
		S2	-	-	-
5.	**F5**	**S1**	-	-	-
		S2	-	-	-
6.	**F6**	**S1**	-	-	-
		S2	-	-	

Note: ‘’F’’ Fraction, ‘’S’’ Spot, ‘’Vis’’ Visible light, ‘’UV’’ Ultra violet light

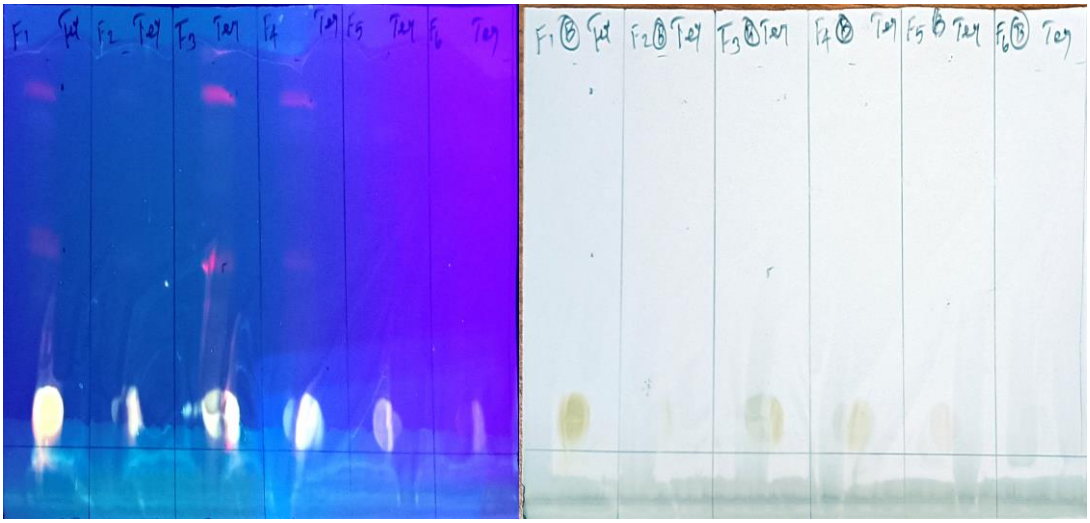
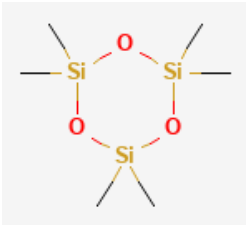


Figure: 4. Detection of Triterpenoids in column fractions of *Costus igneus* TLC by UV light

Spectral Identification of Cyclotrisiloxane, hexamethyl-

The eluted fraction-1 was studied for the presence of selected Cyclotrisiloxane and hexamethyl-compounds using the sample parameters used as before. The compound Cyclotrisiloxane, hexamethyl-was detected at 17.121 retention time in minutes (**Table: 6**). On the other hand, fraction-1 was employed for the structural characterization using H^1 and C^{13} NMR. The peaks obtained from H1 at 18.00 ppm denote the proportions of hydrogen atoms in fraction-1 (**Figure: 6**) while in C13, the spectral graph showed a 1.42 ppm peak representing the carbon skeleton of Cyclotrisiloxane, hexamethyl-and the structure was predicted (**Figure: 7**).

Table: 6. Identification Cyclotrisiloxane, hexamethyl Compounds from fraction-1 by GC-MS

S. No	Chemical Compounds	Retention Time (min)	CAS Number	Molecular Formula	Molecular Structure	Biological Activity
1	Cyclotrisiloxane, hexamethyl-	17.121	000541-05-9	$C_6H_{18}O_3Si_3$		Anti-microbial and Anti-oxidant (S.R, Anjukrishna. *et al*., 2015)

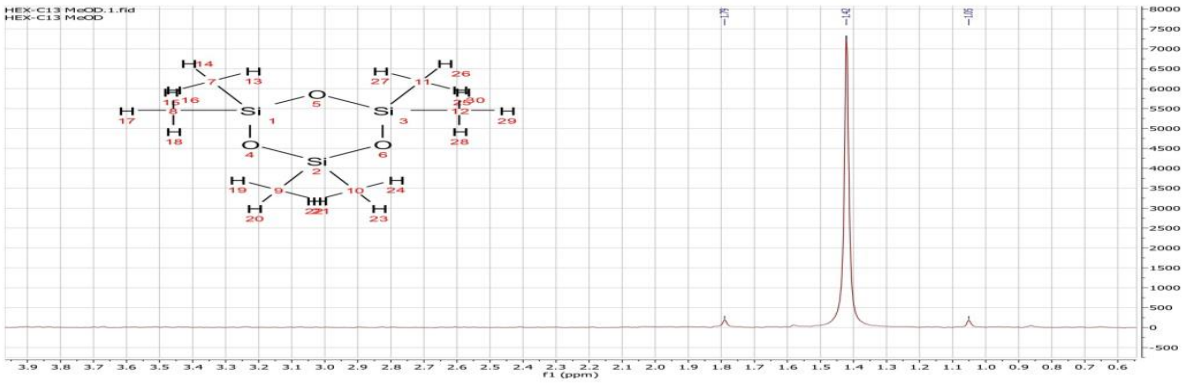


Figure: 6. C[13] NMR spectrum of Cyclotrisiloxane, hexamethyl- from fraction-1 of *Costus igneus*

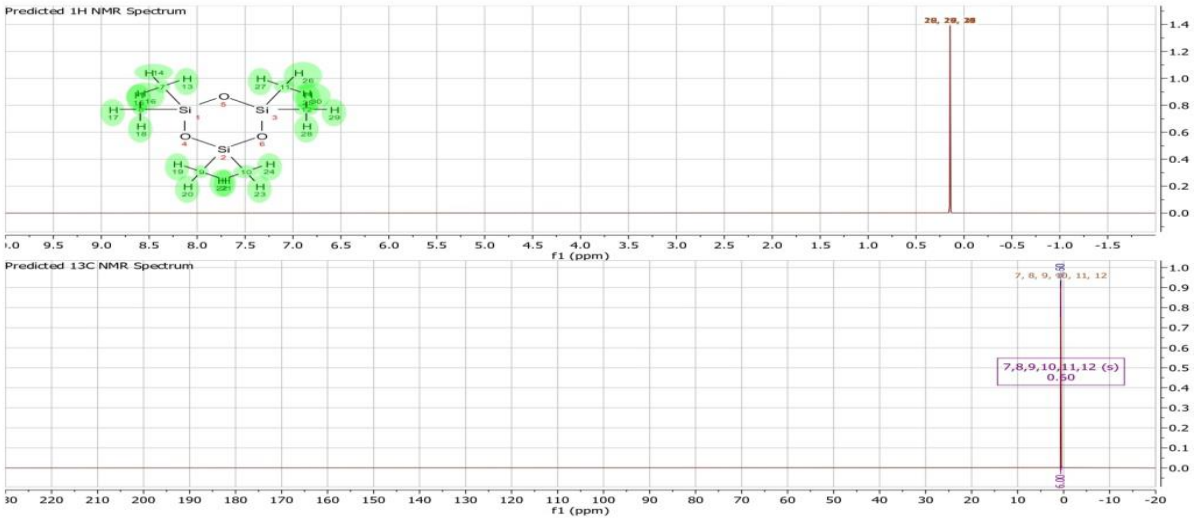


Figure: 7. H[1] NMR spectrum of Cyclotrisiloxane, hexamethyl- from fraction-1 of *Costus igneus*

Discussion

Bio-components are present in various plant parts like leaves, stems, and rhizomes and roots. Several phytochemicals like flavonoids, alkaloids, and terpenoids have been used to treat diabetes in India for thousands of years (Saraswathi R. *et al*., 2017; Kalailingam P. *et al*., 2013). In leaves of *Costus igneus*, the presence of carbohydrates, proteins, alkaloids, tannins, saponins, and flavonoids, rose oxide, fatty acids like hexadecanoic acid, ethyl oleate, oleic acid, and squalene (Radha A. *et al*. 2015). The presence of tannins, phlobatannins, saponins, steroid, terpenoids, and cardiac glycosides in ethanol and methanol extracts of leaves was reported by WHO (2002). From the preliminary screening of phytoconstituents of *Costus igneus* leaves with different polar and non-polar solvents, the prominent presence of triterpenoids in polar (Ethanol, acetone, and DMSO) and non-polar (Diethyl ether and Hexane). The qualitative presence of triterpenoids was also confirmed with an Rf value of 0.79 and triterpenoids were quantified at a concentration of about 91.52 percent in ethanolic extract.

The GC-MS data exposed the presence of bioactive compounds with respective retention time and the compounds were reviewed with their biological potential as followed Methyl salicylate (Aromatic carboxylic acid ester) exhibited anti-Microbial, anti-oxidant and anti-cancer

(Essien, E. E.,2015) and Anti-inflammatory (Li J., 2016). Diethyl Phthalate (Aromatic carboxylic acid ester) showed antimicrobial, acetylcholinesterase, and neurotoxic (Velanganni J., 2011), Cyclotrisiloxane, hexamethyl- (Cyclic hydrocarbon) possessed Anti-microbial and Anti-oxidant (S.R, Anjukrishna. et al., 2015), Thieno[2,3-c]furan-3-carbonitrile, 2-amino-4,6-dihydro-4,4,6,6-tetramethyl- exhibits Analgesic, Antianginal, non-opioid, Antihypertensive, Antiarthritic, Dementia treatment, Neurotransmitter uptake inhibitor (Brintha S. *et al*., 2017) and N-Methyl-1-adamantaneacetamide rewied for their Antimicrobial (Rateb, H.S. *et al*., 2016) and COX-2 Inhibitor (Kakarla, Lavanya *et al*., 2014).

Among these GC-MS identified compounds Cyclotrisiloxane, hexamethyl- (Cyclic hydrocarbon) compound was selected for purification and characterization. The crude ethanolic leaf extract was eluted on sillica column and fractionated. Fraction-1 was further charcterized for the presence of Cyclotrisiloxane, hexamethyl- using GC-MS and H^1, C^{13} NMR. The spectral data revealed the presence and structural confirmation of Cyclotrisiloxane, hexamethyl- compound.

Conclusion

Costus igneus as commonly called as 'Insulin plant' as for their potential activities over wide range of diseases and disorders. The different plant parts of *Costus igneus* were extensively used for traditional medicines and dietary supplements for diabetic mellitus. This study discovered the suitable solvent extraction of phytoconstituents qualitative and quantitatively and identified bioactive compounds through GC-MS and their structural confirmation by proton and carbon NMR.

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