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Pharmacognostic, fluorescent, antibacterial and phytochemical analysis of tuber of *Dioscorea bulbifera* L. from Jharkhand

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Abstract
Dioscorea bulbifera, locally called as 'Githi kanda' and belonging to Dioscoreaceae family, is used as food and ethnomedicine by the tribals of Jharkhand. According to the informants, the tuber powder is used against diarrhoea, malaria and diabetes. Phytochemical screening of the tuber extracts exhibited the presence of alkaloids, starch, coumarin, flavonoids, steroids, terpenoids, cardiac glycosides, phenols, tannins and free amino acids in high concentrations. Analytical HPLC chromatogram revealed only a few bioactive compounds but in good concentrations. GC-MS analysis detected the presence of only five bioactive compounds. Fluorescent study exhibited characteristic colour data while the pharmacognostic evaluation indicated the storage of high amount of starch grains. The present study is supportive of the ethnomedicinal usage of tuber of *D. bulbifera* for the treatment of malaria, diarrhoea, aphrodisiac, rejuvenating and diabetes which may give lead to further the research in isolation and purification of noble drugs for the treatment of given diseases.

Keywords: *Dioscorea bulbifera,* Pharmacognosy, Fluorescence, Phytochemical, HPLC, GC-MS, Antibacterial, Jharkhand

Introduction
Dioscorea bulbifera L. belonging to the family Dioscoreaceae locally called as 'Gitthi kanda' by the tribals of Jharkhand, is commonly found in the hills and forests of Jharkhand. It is a vigorously twining herbaceous vine, with underground and aerial tubers in leaf axils. It grows profusely during the monsoon and leaves behind dried stems in the summer. The tribals gather the tuber as one of their food supplements in the rural areas. According to the informants, the powder of the tuber is used for the treatment of malaria, diarrhoea and diabetes. Pharmacological reports indicate that the tuber possess several medicinal properties such as purgative, deflatulent, aphrodisiac, rejuvenating, anthelmintic, anti-haematological disorders, scrofula, syphilis, anti-haemorrhoids, anti-flatulence, anti-diarrhoea and dysentery, anti-diabetic and anti-polyuria [1]. Pounded tuber is applied on the swellings, boils and ulcers while the roasted tubers are used in dysentery, piles and venereal sores [2]. Ghosh *et al.* (2015) have reviewed 66 therapeutic applications of *D. bulbifera* and presence of 128 phytochemicals in the plant from China and African countries [3].
However, literature survey does not indicate any work on the tuber of *D. bulbifera* from Jharkhand, especially from the pharmacognostic, fluorescent, antimicrobial and phytochemical perspectives, which resulted the present work.

2. Material and Method
2.1 Collection of Plant Material
The plant materials of *D. bulbifera* for the voucher specimens and the tubers for the experiments were collected from Chhipadohar jungles of Latehar district, Jharkhand. The plant materials were identified and deposited in the Rapinat Herbarium of St. Joseph's College, Trichy, Tamilnadu, India under the accession number RHT67248. The different habits and tubers of *D. bulbifera* were photographed and deposited in the same herbarium (Fig. 1).

2.2 Extraction of Phytochemicals
The Tuber of *D. bulbifera* was cut into pieces and dried under shade at room temperature for two weeks. The dried plant material was powdered and kept in an air-tight container.

10g of the powder was extracted in a rotary shaker for 48 hours with 95% ethanol, methanol and distilled water. The extracts were filtered and the filtrate was concentrated and dried by evaporation. Freshly prepared extracts were used for all other analyses.

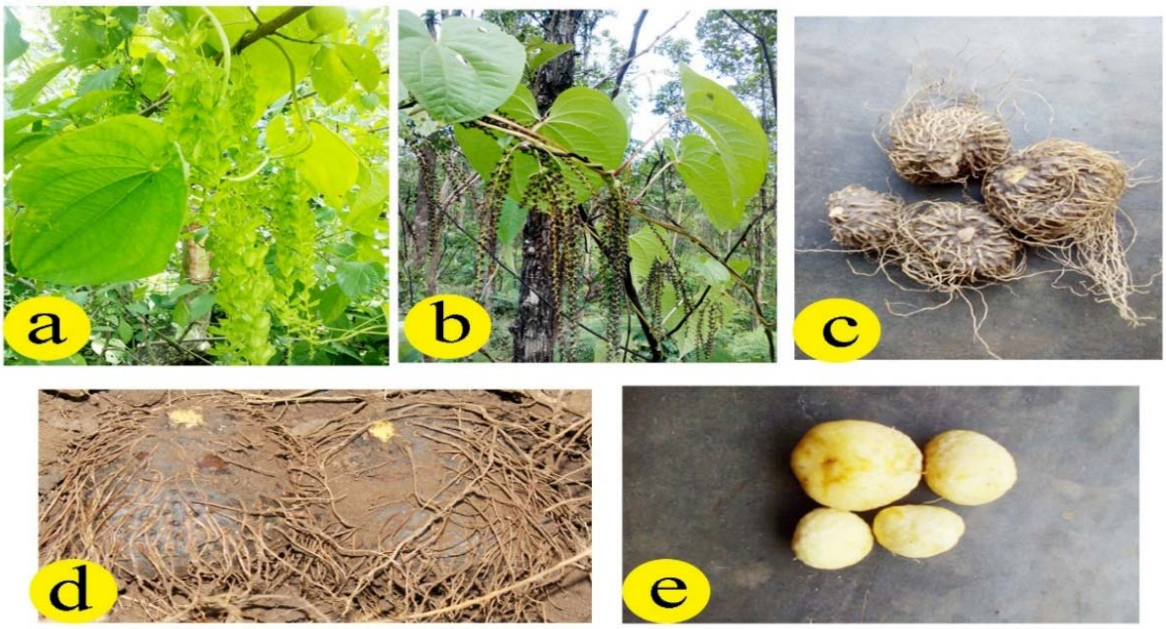


Fig.1: a-b-Habit of *D. bulbifera*; c-d-Tubers; e-Exomorphic features of peeled tubers

2.3 Preliminary Phytochemical Investigations

The behaviour of powder of the tuber of *D. bulbifera* and their basic phytochemical investigations were carried out by adopting the standard methods [4-6].

Qualitative phytochemical analysis of ethanolic, methanolic and aqueous extracts of tuber of *D. bulbifera* was carried out by adopting standard methods from various sources [7-12]. The bioactive compounds such as alkaloids, carbohydrates, flavonoids, glycosides, phenols, saponins, tannins, terpenoids, anthraquinones, etc. were screened by doing three tests for each phytochemical to ascertain the presence.

2.4 Microscopy of Sections and Powder

Thin sections for observation were prepared by standard freehand sectioning and were stained with safranin followed by addition of a few drops of glycerine and covering with a coverslip. The sections were observed under microscope and photographed with Nikon Eclipse 80i.

A pinch of fine powder was taken on a slide and added with a drop of safranin and glycerine. It was then observed under microscope and photographed with Nikon Eclipse 80i. The images of sections and powder microscopy were edited with NIS Elements F 3.00 SP7 and Adobe Photoshop CS6 softwares.

2.5 Fluorescent Study of Power and Extracts

A pinch of the fine powder was taken on a slide and added with a few drops of different chemical reagents such as strong acids and strong bases. The characteristic colour produced due to different reactions were observed and recorded as per the standard methods [4-5].

A small quantity (1ml) of the extract placed on a watch glass and was placed inside the UV viewer chamber and viewed in visible light and short ultraviolet radiations (254 nm). The extracts were observed under the visible light and UV light for their characteristic colour reactions and were compared with a standard colour chart and colour data were recorded [13].

2.6 Antibacterial Activities of Extracts

Twelve bacterial strains consisting of four Gram^{+ve} and eight Gram^{-ve} were selected for the study. The bacterial species were *Bacillus cereus, Bacillus subtilis, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Staphylococcus aureus, Streptococcus pneumoniae* and *Vibrio cholera.* The disc diffusion method was adopted for antibacterial studies in Nutrient agar medium. The concentrations of ethanolic, methanolic and aqueous extracts were 200µg/disc and the same concentration was taken also for the control (streptomycin). The experiments were carried out in triplicates, and the mean and standard deviations were calculated by using standard formulae.

2.7 High Performance Liquid Chromatography (HPLC)

Analytical HPLC was carried out on ethanolic extract of tuber of *D. bulbifera.* Following conditions were applied during the entire process: 2 ml of extract was filtered through 0.2µm filter and 20µl was injected into the Shimadzu HPLC equipped with auto-sampler and diode array detector; solvents used for gradient elution - Acetonitrile and HPLC grade water; running time 30 minutes; chromatograms obtained at 254nm using LC Solution V1.24 software. HPLC analysis was directly performed on ethanolic extract of the tuber of *D. bulbifera.*

2.8 Gas Chromatography—Mass Spectroscopy (GC-MS)

GC-MS analysis of ethanolic and methanolic extracts of *D. bulbifera* was done on GC-MS Shimadzu instrument with following conditions – injection of 4.0 µl of sample; flow rate of helium set to 1.5 ml/min; samples running time about 45 minutes; mass spectra recorded at the range 0-8500 m/z. Identification of compounds were based on comparison of their mass spectra. Interpretation of mass spectrum GC-MS was done using the database of National Institute Standard and Technology research library. The spectra of unknown compounds ware compared with the spectrum of known

compounds stored in the NIST library. The names, molecular formula, molecular weight and molecular structures of the compounds of the test extracts were ascertained from the databank of Chem Spider [14].

3. Results and Discussion

3.1 Behaviour of Powder

The tuber powder of *D. bulbifera* was treated with different chemical reagents and the behaviours of the powders were observed, based on which the inferences were drawn for the presence of the phytochemicals (Table 1). The powder studies indicated the presence of starch phenols, tannins, flavonoids, alkaloids and steroids, and the absence of proteins, quinone and anthraquinone.

Table 1: Behaviour of tuber powder of *D. bulbifera* with chemical reagents

S. N.	Chemical tests	Observation	Inference
1	Powder + Conc. HCl	No yellow colour	Quinone absent
2	Powder + Conc. H_2SO_4	Reddish brown colour	Steroids present
3	Powder + Conc. HNO_3	No yellow colour	Proteins absent
4	Powder + Picric acid	Yellow colour	Alkaloids present
5	Powder + Aq. $FeCl_3$	Light brown colour	Phenols & tannins present
6	Powder + I_2 solution	Dark blue colour	Starch present
7	Powder + NH_3 solution	No blood red colour	Anthraquinone absent
8	Powder + Aq. KOH	No yellow colour	Anthraquinone absent
9	Powder + Aq. NaOH	Pale yellow colour	Flavonoids present

3.2 Fluorescent Analysis of Powder and Extracts

The fine powder was added with different acids and bases, and was observed under visible and UV light (254nm) whose colour data are presented in Table 2. The extracts were observed under different lights without adding of any reagents.

Table 2: Fluorescent analysis of tuber powder of *D. bulbifera* with different reagents

S. N.	Chemical reagents	Visible light	UV light (254nm)
1	Powder as it	Pale brown	Shiny sand
2	Powder + Acetic acid	Pale brown	Brownish yellow
3	Powder + Pot. Dichromate	Golden yellow	Dark brown
4	Powder + Picric acid	Yellow	Brown
5	Powder + Conc. HCl	Pale brown	Dark brown
6	Powder + Conc. H_2SO_4	Blackish brown	Reddish brown
7	Powder + Conc. HNO_3	Pale brown	Reddish brown
8	Powder + I_2 solution	Dark blue	Brown
9	Powder + NH_3 solution	Pale yellow	Golden yellow
10	Powder + Aq. $FeCl_3$	Yellowish brown	Yellowish brown
11	Powder + Aq. KOH	Yellowish brown	Yellowish brown
12	Powder + Aq. NaOH	Pale yellow	Light brown
13	Methanolic extract	Golden yellow	creamy
14	Ethanolic extract	Golden yellow	Creamy
15	Aqueous extract	Creamy	Light brown

3.3 Phytochemical Analysis

Preliminary phytochemical screening of the ethanolic, methanolic and aqueous extracts of tuber of *D. bulbifera* was carried out whose results are presented in Table 3. The extracts were tested for the presence of alkaloids, carbohydrates, flavonoids, glycosides, phenols, steroids, tannins, saponins etc. The data in the table indicate that the highest concentration of bioactive compounds are present in methanolic extract followed by ethanolic and aqueous extracts. Some phytochemicals like coumarin, steroids and terpenoids which were present in organic solvents, were completely absent in the aqueous extract. Moreover, proteins were absent in all the extracts.

Table 3: Phytochemical screening of tuber extracts of *D. bulbifera*

S.N.	Phytochemicals	Methanolic extract	Ethanolic extract	Aqueous extract
1	Alkaloids	++	++	++
2	Carbohydrates	++++	+++	++++
3	Coumarin	++	++	-
4	Flavonoids	++++	++	++
5	Glycosides	++++	+++	+
6	Cardiac glycosides	++++	+++	+
7	Phenols	++++	+++	++
8	Proteins	-	-	-
9	Free amino acids	++++	++++	-
10	Saponins	-	+	+
11	Starch	++	++	+++++
12	Steroids	+++	++	-
13	Tannins	++++	+++	++
14	Terpenoids	+++	++	-

Very high (++++), high (+++), moderate (++), low (+) and nil (–)

3.4 Pharmacognostic Evaluations

3.4.1 Macroscopic Examination

The axillary bulbils were found to be sessile, spherical or oval, and greenish brown when young. The mature bulbils were found to be ash-brown with warty surface and clustered semi-spherical nodules, each nodule with a nipple. The flesh of the bulbils was creamy to pale yellow in colour and bitter in taste. The underground tubers were of different shapes and sizes. The shapes varied from round to oval and oblong. The sizes varied from 250g to 1000g. The tuber skin is purplish brown or soil coloured coated with numerous feeding roots (Fig. 1c-d). The feeding are fibrous and strong. The flesh of the tuber is yellow with characteristic odour and bitter in taste. The peeled tubers are pale yellow in colour and mucilaginous (Fig. 1e). The cut pieces of the tuber are quite sticky due high content of mucilage.

3.4.2 Microscopy of Sections and Powder

Microscopic study of cross sections of the peeled tuber exhibited a darker region, an endodermal layer and lighter region of parenchymatous ground tissue (Fig. 2a-f). Endodermal layers are made up of compact rectangular cells while the ground tissue of polygonal cells. Both the tissues exhibited abundance of starch grains which are triangular with rounded angles. Some starch grains are thick-rod shaped with blunt ends while others oval shaped. They are fewer in number in the cortex regions and densely packed in the ground tissues. Vascular bundles are scattered and fewer in number.

Microscopic examination of the fine powder of *D. bulbifera* showed mostly starch grains with various shapes such as triangular with blunt ends, rod and oval shaped (Fig. 3a-c). The powder also exhibited cell debris and other unidentifiable materials.

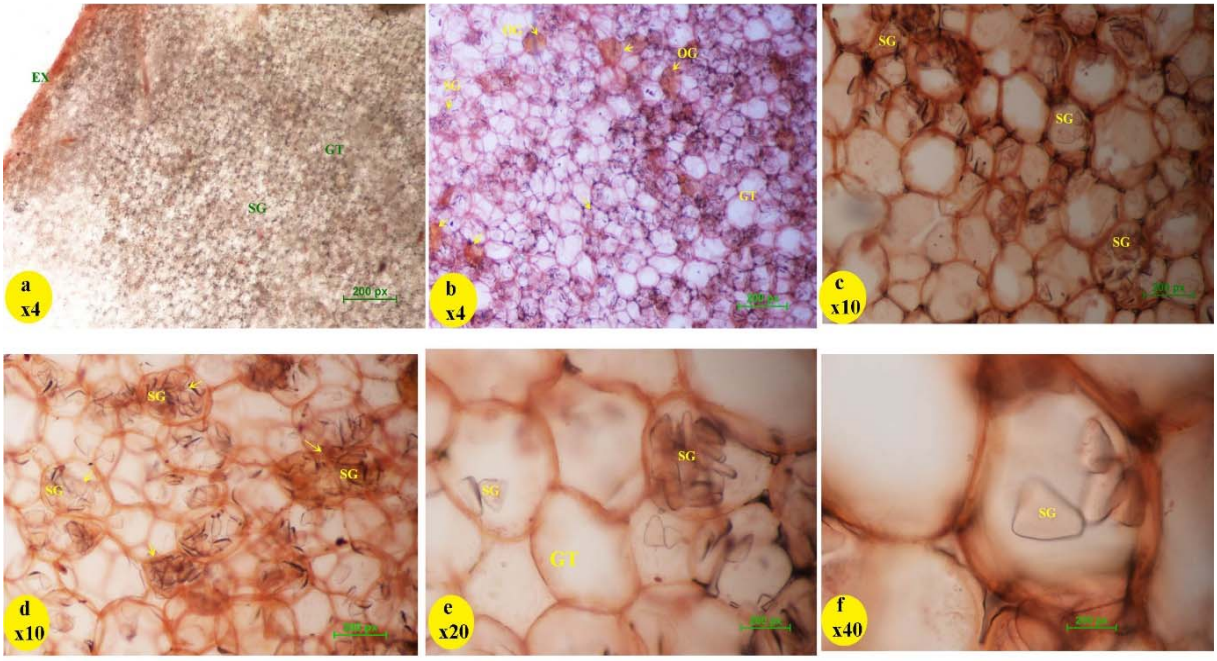


Fig. 2: T.S. of Tuber of *D. bulbifera* - a) A portion in x4; b) A portion of ground tissue in x4; c) A portion of ground tissue in x10; d) A portion in x10 with parenchyma cells; e) A portion showing different shapes of strach grain; f) Triangular starch grains in a cell

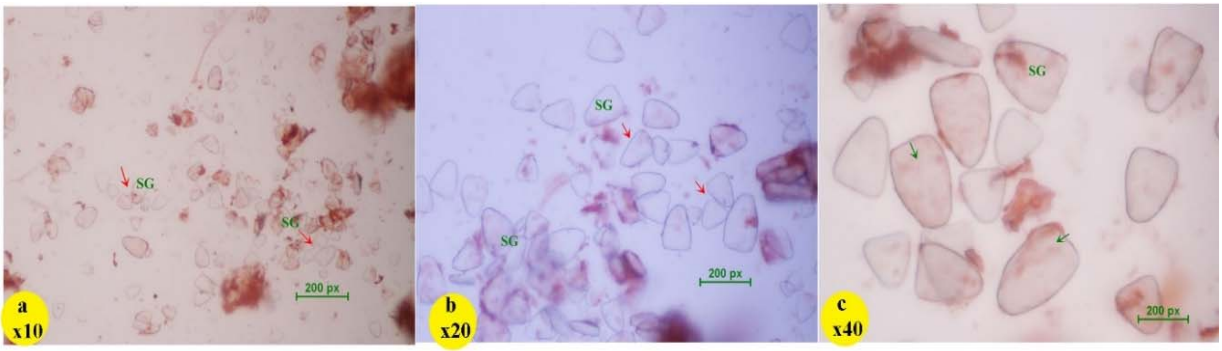


Fig. 3: Powder microscopy of Tuber of *D. bulbifera* - a) View in x10 showing numerous starch grains; b) View in x20 showing different shapes of starch grains; c) x40 view showing triangular, oval and rod shaped starch grains

3.5 Antibacterial Activities

The antibacterial activities of the ethanolic, methanolic and aqueous tuber extracts of *D. bulbifera* were tested against four Gram positive bacteria and eight Gram negative bacteria using Streptomycin as control. The results are presented in Chart 1. All the extracts were ineffective against *B. cereus* and *B. subtilis.* The study revealed that ethanolic and methanolic crude extracts possess higher antibacterial activities than the aqueous extract. Methanolic extract exhibited almost equal inhibition zones in eight human pathogens. The higher inhibition zones produced by ethanolic and methanolic extracts were against *E. aerogenes, E. coli, K. pneumoniae, P. aeruginosa, P. mirabilis, P. vulgaris, S. paratyphi, S. aureus, S. pneumoniae* and *V. cholerae.* Aqueous extract showed minimum activities against the same pathogens.

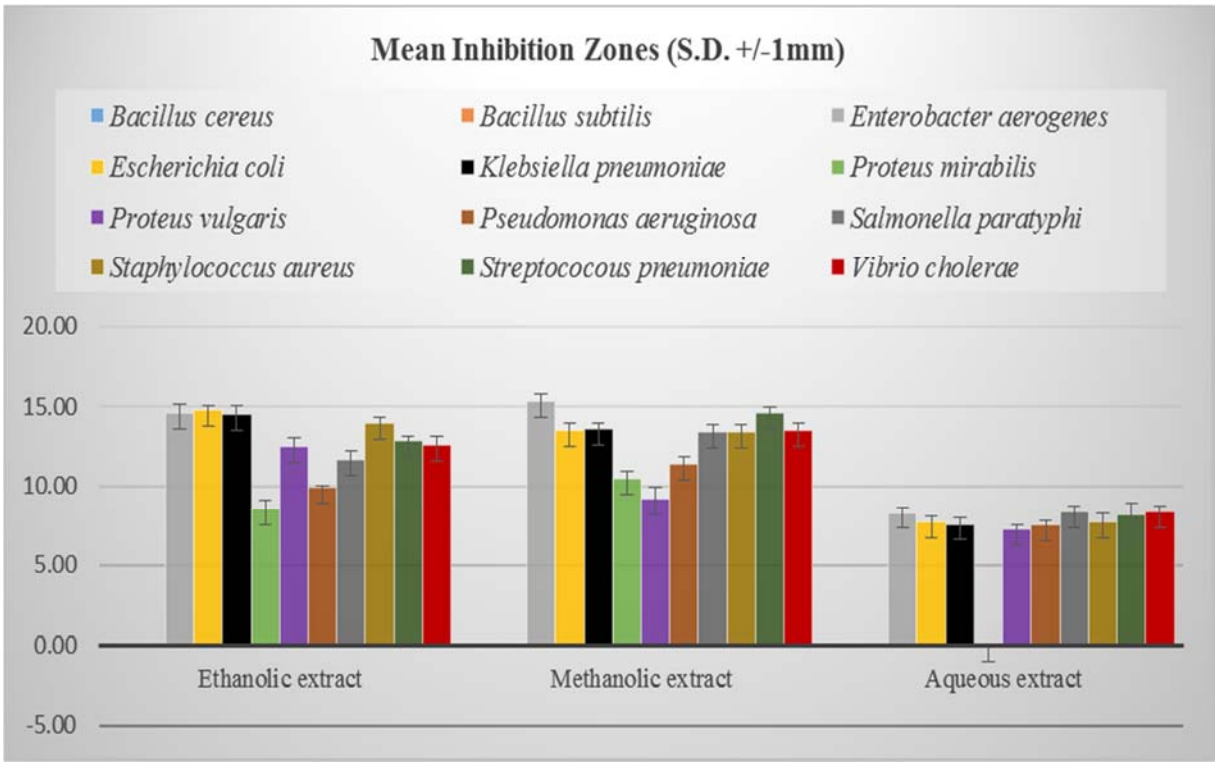
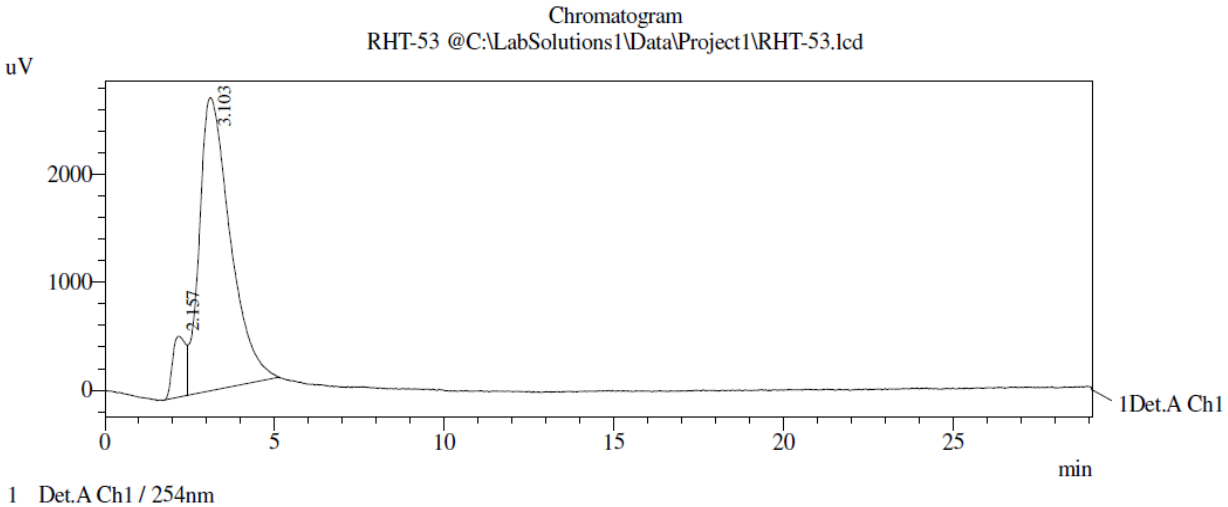


Chart 1: Antibacterial activities of tuber extracts of *D. bulbifera*

3.6 HPLC Analytical Examination



1 Det.A Ch1 / 254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.157	15085	563	8.049	17.152
2	3.103	172331	2718	91.951	82.848
Total		187415	3281	100.000	100.000

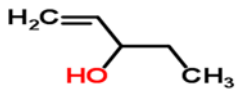
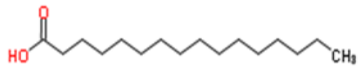
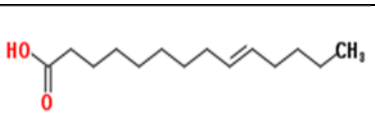
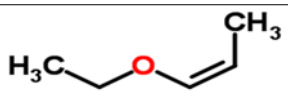
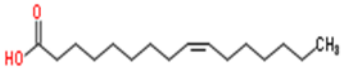
Fig. 4: Analytical HPLC of tuber extract of *D. bulbifera*

The HPLC analysis of ethanolic extract of *D. bulbifera* produced two peaks with retention time 2.157 and 3.103 with area of 8.049% and 91.951% indicating high concentration of two bioactive compounds (Fig. 4). The second peak showing greater 91% of area coverage is an indicative of the presence of highest concentration in the tuber of *D. bulbifera*.

3.7 GC-MS Analysis

The bioactive compounds detected through GC-MS analysis of ethanolic and methanolic tuber extracts of *D. bulbifera* are presented in Table 4. A total of 5 important bioactive compounds were detected in which Palmitic acid was found in both, ethanolic and methanolic extracts consisting of higher area % amounting to 38.54 and 34.99 respectively. Other compounds with their area % are - 1-Ethylallyl alcohol, 21.80; Myristoleate, 39.66; 1-Ethoxypropene, 9.42 and Palmitoleic acid, 55.59.

Table 4: Compounds detected in tuber extracts of *D. bulbifera* using GC-MS analysis.

Peak No.	Ret. Time	Compound name (Common name)	*Molecular formula*	*Molecular weight*	*Molecular structure*	*Area %*
Ethanolic extract		1-Penten-3-ol (*1-Ethylallyl alcohol*)	$C_5H_{10}O$	86.132		21.80
1	12.316					
2	23.308	n-Hexadecanoic acid (*Palmitic acid*)	$C_{16}H_{32}O_2$	256.424		38.54
3	26.575	E-9-Tetradecenoic acid (*Myristoleate*)	$C_{14}H_{26}O_2$	226.355		39.66
Methanolic extract		Ethyl-1-propenyl ether (*1-Ethoxypropene*)	$C_5H_{10}O$	86.132		9.42
1	12.814					
2	23.348	n-Hexadecanoic acid (*Palmitic acid*)	Given above	Given above	Given above	34.99
3	26.604	9-Hexadecenoic acid (*Palmitoleic acid*)	$C_{16}H_{30}O_2$	254.408		55.59

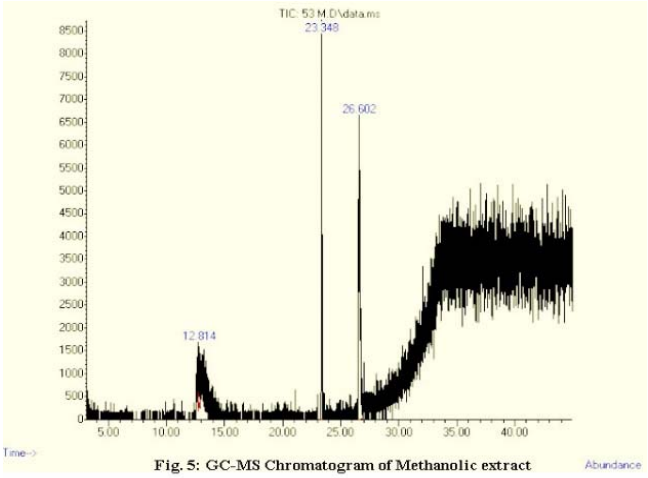


Fig. 5: GC-MS Chromatogram of Methanolic extract

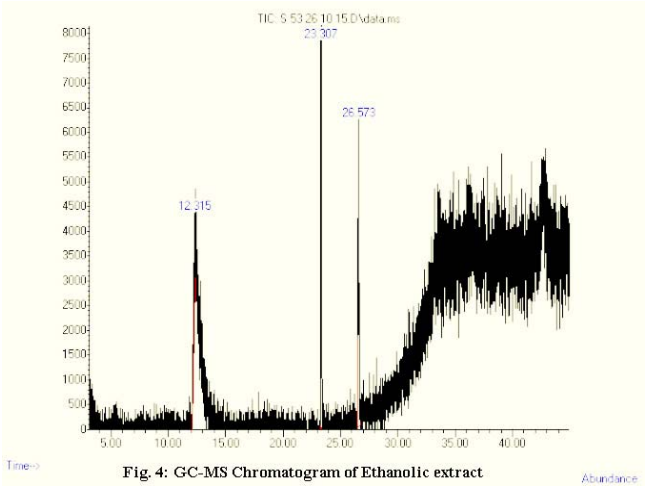


Fig. 4: GC-MS Chromatogram of Ethanolic extract

4. Conclusion

Preliminary phytochemical screening exhibited the presence of higher concentration of bioactive components in methanolic extract of tuber of *D. bulbifera* followed by ethanolic extract. The aqueous extract showed only a minimum concentration. The HPLC analysis exhibited the presence of two major compounds while GC-MS analysis revealed the presence of five major bioactive compounds. Methanolic extract contributed to higher antibacterial activities followed by ethanolic extract. It is interesting to note that the aqueous extract did not show any antimicrobial activities. The tuber possess potent antibacterial activities against gastrointestinal pathogens which validates the indigenous usage of the tuber against diarrhoea dysentery. Its ethnomedicinal usage against diabetes seems to be for the management and not for the treatment as the tuber is very rich in starch grains. Hence, the present study is supportive of the ethnomedicinal usage of tuber of *D. bulbifera* for the treatment of malaria, diarrhoea, aphrodisiac, rejuvenating and diabetes. Moreover, it could give lead to further the research in isolation and purification of noble drugs for the treatment of given diseases.

5. Acknowledgments

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