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GC-MS Determination of bioactive compounds constituents of freshwater edible snail (*Bellamaya bengalensis)*

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Abstract
This work was a structured screening for the bioactive compound from edible snail *Bellamaya Bengalensis*. The present study aims at isolating the bioactive components using Gas Chromatography-Mass Spectroscopy (GC-MS). The Mt COI gene sequenced isolated and submitted in NCBI to obtain the accession number (MT089706) of *Bellamaya bengalensis* from NCBI. The bioactive components of edible snail have been evaluated using GC-MS and a total of 42 bioactive compounds are identified. The compound with least retention time (3.052) is identified as glycoaldehyde dimer and the highest retention time (29.529) taken to isolate is the compound 6-octadecanoic acid, methylester. The FTIR analysis confirmed the presence of alcohol, ether, amino, inorganic ions and saturated aliphatic functional groups. The identified compounds are known to exhibit medicinal values.

Keywords: *Bellamaya bengalensis,* bioactive compounds, DNA barcoding

Introduction
Mollusca are considered as a vital variety of shell fishes; Gastropod is an ecologically important class of phylum mollusca which forms an important link in the food web of different ecosystems and is an excellent biological indicator for environmental quality (Santhiya and Ramasamy, 2019; Li and Gao, 2014) [1, 2]. Snails are considered as a delicacy since immemorial times both in developed and less developed countries of the world and are distributed all over Asia and Africa (DeMarco *et al*., 2017; Robert *et al*., 2013) [3, 4]. It is widely distributed in all parts of India and is especially enjoyed by the ethnic races of North East Indian states and also in other states like Punjab, Bihar, Maharashtra, and Jharkhand. It is enjoyed as a source of food because of its high nutrition and culinary value (Moniruzzaman *et al*., 2021; Fernández *et al*., 2017) [5, 6].
Bellamaya bengalensis have 16-25 mm long calcareous shells, closed with an opercula attached with foot muscles, which is the main edible part (Baby *et al*., 2010) [7]. Many studies have reported that edible mollusca are highly nutritious and are salutary due to its rich source of essential amino acids, low fat concentration and a good source of vitamins, minerals and essential fatty acids (Pissia*et al*.,2021; Khan *et al*., 2019; Ahmad *et al*.,2018) [8, 9, 10]. Snail meat is rich in bioactive components and these compounds are also known to posses' therapeutic potential by reducing oxidative stress, inflammation and treating many metabolic disorders (Siriwardhana *et al*., 2012) [11]. Bioactive components are characterized as antimicrobial, antifungal, sedative, and antioxidant which are known to target innate immunity, thereby improving defense mechanism (Dhiman *et al*., 2021) [12]. Antioxidants are chemical compounds which have the ability to reduce free radicals and thus help in decreasing lipid per oxidation that can lead to various diseases and aging (Galati *et al*., 2004) [13]. They are a store house of medicinal properties and are used in the treatment of viral lesions, warts, skin problems, treatment of Alzheimer, dementia, hypertension, wound healing, arthritis, cardiac arrest, stroke (El-Zawawy *et al*.,2021; Harti *et al*.,2016) [14, 15]. Thus, the aim of the present work was to identify the bioactive compounds constituents with aid to GCMS.

Materials and Method
1. Sample Collection
Bellamaya bengalensis species was collected from local paddy field and fishery ponds in Dimapur district, Nagaland, India (Sample collected sit Apporxment Temprature July 29° C (85.5 F). The live specimens were collected by hand picking method and transported to the lab within 24 hours.

2. Methanolic Extraction
The soft bodies were removed by breaking the shell, washed thoroughly in distilled water and dried at 60^0C overnight using hot air oven and powdered. Methanol extracts was prepared by the addition of absolute Methanol (Analytic CSS Reagents) (20 ml) to 2.0 g of the powdered samples of the two species and left undisturbed for 72 h. The samples were then filtered using filter paper, Whatman No. 1. The filtrates from the filtration procedure were left to evaporate at room temperature. The dried residues (extracted from soft bodies

tissue) (50 mg) were re-dissolved in 20 ml of absolute methanol and stored at 4^0C until further use for different analysis (De *et al*., 2010) [16].

3. DNA Barcode

The freshwater gastropod and bivalve was identified morphologically using standard literature (Ramakrishna *et al*., 2007; Rao 1989) [17, 18]. The specimens were thoroughly cleaned body tissue was removed and preserved in 99.9% ethanol. DNA was extracted from the body tissue using CTAB extraction or Kit-based methods (NucleoSpin Tissue Kit (Macherey-Nagel). The genes were amplified using PCR performed in 25 µl volumes that contained 1x Taq buffer, 200µM 1U Taq polymerase, 1,5Mm $MgCl_2$ and 200µM Dntp.

Standard primers used were LCO 1490 5'-GCTCAACAAATCATAAAGATATT-3'and HCO2198 var. 5'-TAWACTTCTGGGTGKCCAAARAAAT-3' (Wilson *et al*., 2004) [19]. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al*., 2010) [20].

4. Fourier Transform Infrared Radiation (FTIR)

The powdered sample obtained from methanolic extract was characterized in a Fourier-transform infrared spectromemter using KBr pellet and recorded with FTIR 460 plus Jasco at a spectral range of 400 to 4000 cm^{-1} and at a resolution of 4 cm^{-1} (Movasaghi *et al*.,2008) [21]. FTIR analysis indicates the type of chemical bonds or functional groups present by analysing the infrared absorption spectrum (Ashokkumar *et al*., 2014) [22]. Each peak is caharcterized by a specific functional group.

5. Gas Chromatography- Mass Spectroscopy

A column of GCMS-QP2010 (Japan) Plus was used. Oven temperature was programmed from 40^0C (for 3 min), which is increased at a rate of 7.5 and then finally programmed at 300^0C for 3 min. The total running time of GC was 40 minutes. Identification of compounds: Interpretation of the compounds present were done using the library database of National Institute Standard and Technology 11.0 (NIST) and Wiley8.LIB.

Result and Discussion

1. DNA Barcoding

DNA extracted from tissue sample was amplified and sequenced up to 450 to 680 base pair length. BLAST was used to check homology between the retrieved sequences in Gene Bank library. Analysis of homology using BLAST revealed that the sample was 86.63 % pair wise similarity with voucher of *Bellamaya bengalensis* (FJ405877) in Gene Bank. The COI gene sequences is submitted in Gene Bank in NCBI to obtain gene bank accession number (MT089706) for *Bellamaya bengalensis* and COI gene sequences is submitted in BOLD system for generation of DNA barcode. Based on the previous research reports, who has been phylogeneitc tree being constructed according to partial COI gene sequence data confirmed that Bellamya litbophaga (Youzh *et al*., 2014) [23].

2. Fourier Transform Infrared Radiation (FTIR) Analysis of *Bellamaya bengalensis*

Results of FTIR spectroscopic studies have revealed the presence of various chemical compounds in the sample. The wave number, absorption bands and the dominant peaks are defined in Figure 4 and Table 1. The absorption bands at 3376 cm^{-1} and 1407 cm^{-1} has been to O-H bond stretching, 2957 cm^{-1}, 1448 cm^{-1}, 2925 cm^{-1} and 2853 cm^{-1} to C-H bond stretching (Methylene),1628 cm^{-1} to C=C bond stretching (Alkeny),1546 cm^{-1} to Aliphatic nitro compounds, 1240 cm^{-1},1079 cm^{-1},1153 cm^{-1} which has been assigned to C-O bond stretching, 1027 cm^{-1} to C-F bond stretching, 932 cm^{-1} and 862 cm^{-1} to indicates for silicate ion and carbonate ion respectively. The most intense band is at 3376.89 cm^{-1} representing the presence of alcohol group (O-H) and the weakest peak at 578.19 cm^{-1} indicating the presence of disulfide functional group (C-S). An intense band occurring at 3376.89 cm $^{-1}$ and 1407.66 cm $^{-1}$ corresponds to normal polymeric OH stretch vibration and OH bend vibration indicating the presence of alcohol and hydroxyl compound. Stretching frequencies are higher than corresponding bending frequencies because it is easier to bend a bond than to stretch or compress it. This FT-IR spectra analysis follows the same pattern as that deduced in a study on the three freshwater snails *Bellamya bengalensis, Pila globosa and Brotia costula* (Ramya *et al*.,2015) [24]. The absorption bands at 2957.65 cm^{-1} and 1448.46 cm $^{-1}$ indicates methyl C-H asym/sym. stretch and bending vibrations respectively and exhibits the presence of saturated aliphatic alkyl group. While the band at 2925.26 cm^{-1}, 2853.33 cm^{-1} exhibited the presence of methylene C-H asym. /sym. stretching indicating the presence of saturated aliphatic methylene group. A C=C stretch bond at peak 1628.64 cm^{-1} indicates the presence of olefinic/alkene group which is a characteristic bond in ascorbic acid (Vitamin C). Aliphatic nitro compound has a peak at 1546.88 cm^{-1} confirming the presence of nitrogen-oxy compound. Relatively weak peaks at 1240.37 cm^{-1} and 1079.18 cm^{-1} indicates the presence of aromatic ether (aryl-O stretch) and alkyl substituted ether (C-0 stretch) which confirmed the presence of ether and oxy compound respectively. A moderately weak peak at 1153.17 cm $^{-1}$ indicates the presence of secondary amine (C-N stretch) which can be considered a diagnostic component for the determination of the various physiological phases of the cell as it indicator for the presence of secondary amino (protein) group (Ramya *et al*.,2015) [24]. The band at 1027.48 cm^{-1} indicates C-F stretch which confirms the presence of aliphatic fluro compounds. The absorption bands at 932.82 cm^{-1} and 862.01 cm^{-1} indicates the presence of inorganic ions (silicate ion and

carbonate ion). A moderately weaker absorption bands at 699.55 cm^{-1}, 578.19 cm^{-1} indicating Aryl thioethers (C-S stretch) and Disulfides (C-S stretch) vibration mode confirms the presence of thiols and thio-substituted compounds. This deduction follows the findings discussed in the case of waste shell dust of freshwater mussel *Lamellidens marginalis* (Parveen *et al*., 2020) [25].Thus, depending on the peak values, the functional groups can be determined.

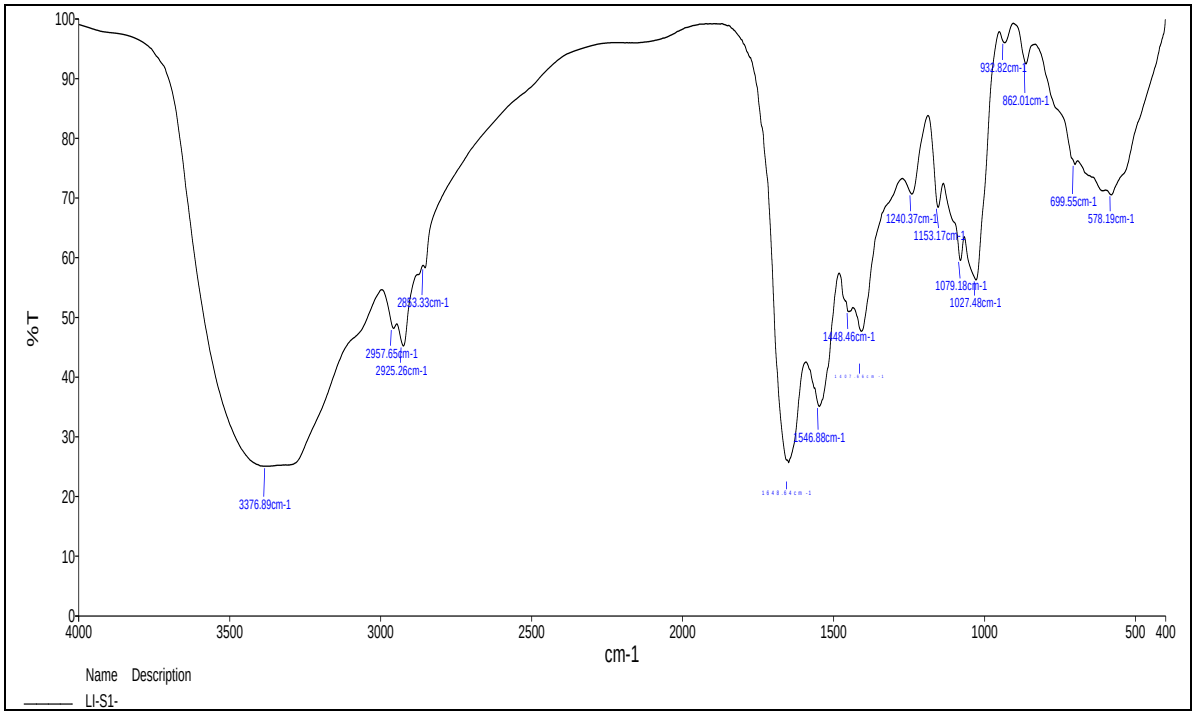

Fig 1: FT-IR spectrum of freshwater Gastropod *Bellamaya-bengalensis*

Table 1: Functional group of freshwater Gastropod *Bellamaya bengalensis* and its quantified frequencies

Wavenumber (cm^{-1})	Vibration mode	Functional group	Wavenumber (cm^{-1}) (ref. Coates, 2000)
3376.89	Normal polymeric OH stretch	Alcohol and hydroxyl compound	3400-3200
1407.66	Phenol or tertiary alcohol, OH bend		1410-1310
2957.65	Methyl C-H asym./sym. Stretch	Saturated aliphatic alkyl Saturated aliphatic methylene	2970-2950
1448.46	Methyl C-H asym./sym. Bend		1470-1430
2925.26	Methylene C-H asym./sym. Stretch		2935-2915
2853.33	Methylene C-H asym./sym. Stretch		2865-2845
1628.64	Alkenyl C=C stretch	Olefinic (alkene)	1680-1620
1546.88	Aliphatic nitro compound	Nitrogen-oxy compound	1540-1560
1240.37	Aromatic ethers, aryl-O stretch	Ether	1270-1230
1079.18	Alkyl-substituted ether, C-O stretch	oxy compound	1150-1050
1153.17	Secondary amine, CN stretch	Secondary amino (protein)	1190-1130
1027.48	C-F stretch	Aliphatic fluoro compound	1150-1000
932.82	Silicate ion	inorganic ions	1100-900
862.01	Carbonate ion		880-860
699.55	Aryl thioethers, Ø-S (C-S stretch)	Thiols and thio-substituded compounds	670-715
578.19	Disulfides (C-S stretch)		705-570

3. Gas Chromatography- Mass Spectroscopy Analysis

The bioactive components present in *Bellamaya bengalensis* was identified and confirmed by GC-MS analysis (Fig. 2). Fig. 2 shows the presence of 42 bioactive components in the sample. The principles with retention time (RT), peak area, molecular formula, molecular weight (MW), biological activity are presented in Table 2. The mass spectra of the biologically active components in the sample are presented in Fig. 3. The compound with least retention time (3.052) is identified as glycoaldehyde dimer and the highest retention time (29.529) taken to isolate is the compound 6-octadecanoic acid, methylester. The height of the peak represents the relative concentration of compounds present and analyzing of the elution of compounds at different times helps in identification of the structure and the nature of the bioactive compounds present (Movasaghi *et al*., 2008) [26].This result is supported by the presence of bioactive compounds in Giant African Snail (*Archachatina marginata*) where 26 compound were isolated from the haemolymph (Lawal *et al*., 2015) [27]. In the preliminay

study, study assessing the biomedical characteristic of such compounds, Furter, utilization for naturally occuring bioactive compound form *Bellamaya bengalensis* could obtain a wide altenative of matufactured therapetics. Interestingly, these findings would be the opening the new trends in biomedical and pharmaceutical industries in the region.

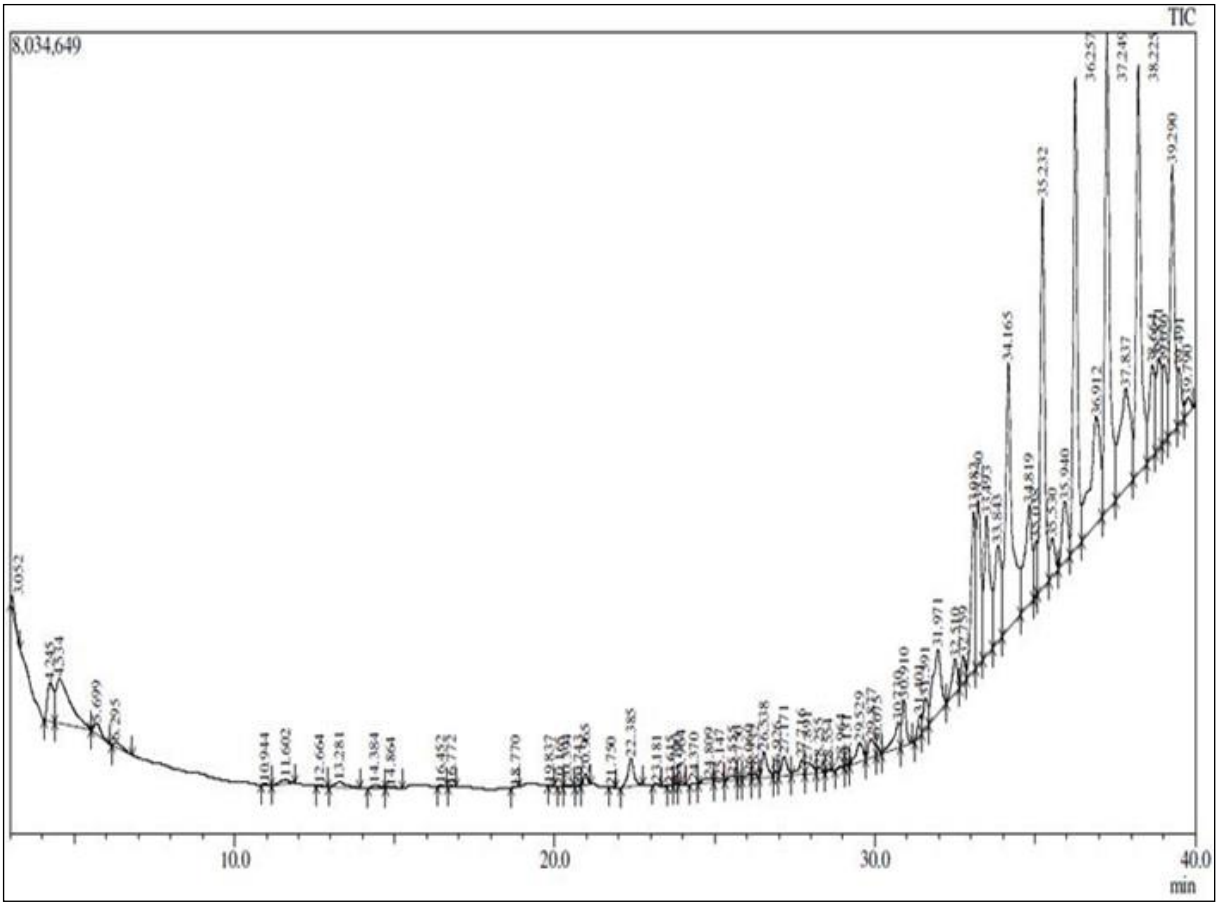

Fig 2: GC-MS analysis of freshwater gastropod *Bellamaya bengalensis*

Table 2: GC-MS analysis of freshwater gastropod *Bellamaya benghalensis*

Peak	Retention time	Compound name	Molecular formula	Molecular weight	Peak area
1	3.052	Glycoaldehyde dimer	$C_4H_8O_4$	120	0.21
2	4.245	Ethanol,2-(dimethylamino)-	$C_4H_{11}N0$	89	1.08
3	4.534	Ethanamine,2-Chloro-N,N-dimethylamine	$C_4H_{10}ClN$	107	2.81
4	5.699	Azetidine,3-Methyl-1-(1-methyethyl)	$C_7H_{15}N$	113	0.32
5	6.295	1 Butanol,2-Amino	$C_4H_{11}NO$	89	0.18
6	10.944	N-cyclohexyl-1,3-propanediamine	$C_9H_{20}N_2$	156	0.03
7	11.602	Pentanoic acid,3-hydroxy-4-methyl-,methyl e	$C_7H_{14}O_3$	146	0.20
8	12.664	3,4-Hexanediol,2,5-Dimethyl-	$C_8H_{18}O_2$	146	0.03
9	13.281	n-octymethylimine	$C_9H_{19}N$	141	0.37
10	14.384	Ethyl-5,5-diethoxy Valerate	$C_{11}H_{22}O_4$	218	0.15
11	14.864	Heptanoyl chloride	$C_7H_{13}ClO$	148	0.08
12	16.452	Tetradecane	$C_{14}H_{30}$	198	0.02
13	16.772	4 pyrimidinamine, 6-methyl	$C_5H_7N_3$	109	0.01
14	18.770	Heptane,2,3- epoxy-	$C_7H_{14}0$	114	0.02
15	19.837	3-Hydroxypropanohydrazide	$C_3H_8N_2O_2$	104	0.01
16	20.165	7-Tetradecen-1-ol, (Z)-, TMS derivative	$C_{17}H_{36}O_3Si_3$	284	0.01
17	20.394	Pyrogallol, 3 TBDMS derivative	$C_{24}H_{48}O_3Si_3$	468	0.06
18	20.743	1-(2,3- Difluro Benzoyl) Azepane	$C_{15}H_{15}F_2NO$	239	0.01
19	20.965	Phenol,2,6-bis (1,1-dimethylethyl)	$C_{14}H_{22}O$	206	0.17
20	21.750	Undecane	$C_{14}H_{24}$	156	0.01

21	22.385	1,2- benzenedicarboxylic acid, DIE	$C_{12}H_{14}O_4$	222	0.75
22	23.181	3-Pentanol,3-Methyl-carbamate	$C_7H_{15}NO_2$	145	0.04
23	23.615	Heptanal	$C_7H_{14}O$	114	0.01
24	23.790	p-octyloxynitrobenzene	$C_{14}H_{21}NO_3$	251	0.02
25	23.964	Nonadecane	$C_{19}H_{40}$	268	0.06
26	24.370	5-Amino-1-methyl-1H-Imidazole-4-c	$C_5H_7N_3O_2$	141	0.21
27	24.809	2-Hexylorixane	$C_8H_{16}O$	128	0.21
28	25.147	1-Nonanol	$C_9H_{20}O$	144	0.12
29	25.555	Glycyl-L-proline	$C_7H_{12}N_2O_3$	172	0.16
30	25.750	Decanoic acid,methyl ester	$C_{11}H_{22}O_2$	186	0.06
31	26.060	Carbonic acid nonylpro-1-en-2-yl ester	$C_{13}H_{24}O_3$	228	0.12
32	26.232	Undecane	$C_{11}H_{24}$	156	0.19
33	26.538	Diisobutyl Benzene-1,2-dicarboxylate	$C_{16}H_{22}O_4$	278	0.59
34	26.926	Dodecanoic acid,3-Hydroxy-	$C_{12}H_{24}O_2$	216	0.08
35	27.171	Ecicosanoic acid, methylester	$C_{21}H_{42}O_2$	326	0.48
36	27.716	Tridecane,4-Methyl	$C_{14}H_3O$	198	0.37
37	27.891	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo	$C_{14}H_{22}N_2O_2$	250	0.37
38	28.255	Hexadecane,5-Butyl	$C_{2O}H_{42}$	282	0.10
39	28.554	Decanoic acid,methyl ester	$C_{11}H_{22}O_2$	186	0.03
40	28.964	Tridecane, 5-Propyl	$C_{16}H_{34}$	226	0.05
41	29.131	1-Hexadecanol,2- Methyl-	$C_{17}H_{36}O$	256	0.02
42	29.529	6-octadecanoic acid, methylester	$C_{19}H_{36}O_{22}$	296	0.60

Conclusion

Therefore, GCMS methods is a direct and fast analytical approache for identificatio of bioactive compounds only few grams of tissue materils is required. The present investigation has identified and confirmed the presence of bioactive components by using FTIR and GCMS analysis in freshwater gastropod *Bellamaya bengalensis*. Thus it can be considered a good source of natural product in human therapy and also potential usage of it in various pharmaceuticals. The bioactive compounds characterizatio of the extracts, the isolatio of responsible bioactive compounds and their biological activity are necessary for future studies.

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