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Isolation and identification of different amino acids and other phytoconstituents through LCMS, GCMS analysis of *Ananas comosus* and evaluation of antifungal activity

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ABSTRACT

Aim-The project investigates the presence of different phytoconstituents and antifungal activities of protein concentrated part of Pineapple, scientifically known as *Ananas comosus*.

Objective-Pineapple scientifically known as *Ananas comosus* having different phytoconstituents unexplored and evaluation of antifungal activity have to be established.

Material and method-The pineapple juice have been extracted and lyophilized. Then ion exchange chromatography was performed to isolate different amino acid. Different salts were used for protein precipitation, and various solvents were used, including petroleum ether, n-hexane, ethanol, methanol, and aqueous media. Then this protein concentrated part was sent to TAAB Biostudy center, Kolkata, aqueous extract was send to IIB Kolkata for LCMS, n-hexane part was sent to GCMS to IICB, Kolkata,

The antifungal activity was performed with A. niger & C.albicans with ethanolis extract and two different protein concentration with Sabogaurd media.

Results-The amino acids glycine, alanine, phenylalanine, glutamic acid, histidine and arginine were found in isolated part of protein concentration. The LCMS study of aq extract of shows the presence of Gallic acid, Pyrocatechol, Hydroxytyrosol, Chlorogenic acid, Caffeic acid, Syringic acid etc. The GCMS study shows the presence of decane, 3,7-dimethyl-; benzene, 1,3-bis(1,1-dimethylethyl)-; dodecane, 4-methyl-; dodecane, 2,6,11-trimethyl-; heptadecane; 2,11-2,6,10-trimethyltridecane; nonadecane etc.

The antifungal activity was established for A. niger & C.albicans with ethanolis extract with ethanolic extract and protein concentrated part I.

INTRODUCTION

Pineapple is a tropical fruit, a member of the Bromeliaceae family. It is mostly consumed raw but can also be processed into juice, drinks, jams, and jellies (1). The Ananas comosus is a herbaceous perennial that may reach heights of 1.0 to 1.5 meters (3.3 to 4.9 feet), while it can occasionally reach much higher heights. The plant itself is visually striking, with a short, stocky stem and stiff, waxy leaves (2). When it blossoms, the separate fruits of the flowers come together to form a pineapple. Commercial growers "Following the first fruit, the main stem's leaf axils produced side shoots, which are known as suckers by Commercial growers(3). Pineapple is one of the most beneficial fruits for producing value-added compounds such as antioxidants, organic333 aci33ds, bromelain, Sesquiterpene, and phenolic compounds, according to its physicochemical makeup and nutritional value (4). Pineapple stems are the source of the potent chemo-responsive proteolytic enzyme bromelain. It is isolated and purified using various techniques and contains several thiol endopeptidases (5). Though scientists have also shown that it has antibacterial and anticancer properties, it is most typically utilized as an anti-inflammatory medication. (6)

Materials and methods

Apparatus used: Beakers, measuring cylinder, conical flask, volumetric flask, petri dish, Buchner funnel, separating funnel, filter paper, aluminum foil, glass rod, pipette, spatula, thermometer, reagent bottle, bubbler, centrifugal tube, Eppendorf tube, tray, knife.

Instrument used: Centrifugal Machine, Electrical weighing balance, pH meter, Water bath, Mixer grinder machine, Hot air oven, Lyophilizer(Labmade),Rotary evaporator(Elab),LC-MS(SCIEX), GC-MS(Perkin elmer), FTIR(bruker),

Chemicals used: Diethyl ether, petroleum ether, ethanol, water, pineapple juice, Polyethylene glycol 1500, $MgSO_4.7H_2O$, Ethylene diamine tetra acetate (EDTA), phosphate buffer, Ammonium acetate, ammonium sulphate.

1. Preparation of juice:

Fresh pineapple (*Ananas comosus*) was purchased from the market (Barasat, West Bengal,India). The pineapple core was removed. Then, the pineapple was sliced, and the peels were removed. The flesh part of the pineapple was chopped and ground using a mixer grinder machine. After the grinding process was completed, the mashed part was filtered through a vacuum filter with the help of a Buchner funnel. The filtrate of pure juice was then collected in a container.

2. Extraction and Lyophilization of juice from pineapple fruit

The pineapple core was ground using a domestic juicer without adding water or buffer, and the contents were centrifuged at 8000×g at 4ºC for 30 minutes. The resulting supernatant was cooled to 4ºC, and proteins were precipitated using ammonium sulphate in the 40–80% saturation [1](Devakate et al., 2009). The salt was gradually added to the extract under constant agitation, and after complete addition, the mixture was stirred for an additional 30 minutes at 4ºC. The resulting suspension was then centrifuged at 8000×g at 4ºC for 15 minutes. The supernatant was discarded, and the precipitate was dissolved in a minimal volume of 25 mM potassium phosphate buffer at pH 7.0. This sample was desalted using a gel filtration column (PD-10, Sephadex G-25 M, GE Healthcare, USA), frozen in liquid nitrogen, and lyophilized following the manufacturer's protocol (Lioalfa 6-80, Telstar, Spain).

3. Purification of protein by ion exchange chromatography

The chromatographic procedure was conducted using a low-pressure chromatographic system (Gilson, France) at room temperature, with a flow rate of 0.5 mL min^{-1} (superficial velocity of 38.2 cm h^{-1}). The ion exchange medium, DEAE-Sepharose, was suspended in water, degassed, and packed without compression into columns (10.0 cm × 1.0 cm I.D., GE Healthcare, USA) to yield a bed

volume of 1.0 mL. The columns were equilibrated with the respective loading buffer. To study the influence of buffer pH on enzyme adsorption, the following loading buffers were used: 25 mM McIlvaine buffer (sodium phosphate dibasic and citric acid) at pH 5.0; 25 mM potassium phosphate at pH 7.0; and 25 mM Tris-HCl at pH 9.0. A pre-purified, freeze-dried bromelain solution (0.3 mg mL^{-1}) was prepared in each buffer and used as the feed solution for column loading. The column was washed with the corresponding loading buffer after sample injection until absorbance at 280 nm approached zero. Elution was carried out by increasing the loading buffer's ionic strength by adding 1.0 mol/L^{-1} NaCl. After each run, the column was regenerated using 50 mM NaOH, followed by rinsing with ultrapure water. Throughout the procedure, fractions of 2.0 mL were collected.

4. Extraction of pineapple juice (Cold maceration):

The pineapple juice was allowed to dissolve in four different solvents: Petroleum ether, Diethyl ether, Ethanol, and Water. Mixing of juice in four different solvents are as follows:

Table 1: Different solvent and their amount mixed with pineapple juice.

Solvents	Amount of juice (ml)	Amount of solvent (ml)	Total volume of mixture (ml)	Ratio of mixture
Diethyl ether	100	100	Up to 200	1:1
Petroleum ether	100	100	Up to 200	1:1
Ethanol	100	100	Up to 200	1:1
Water	100	100	Up to 200	1:1

Then The extracted juice was allowed to cool in the refrigerator at 2-8˚C for 3-4 days due to elevated temperature; the phytochemicals present in the juice can degrade.

5. Collection and analysis of extracts:

After 3-4 days, two separate layers were formed in each mixture, containing organic and aqueous layers. These two layers are separated through a separating funnel for each mixture. Diethyl ether and petroleum ether are highly volatile organic compounds; hence, they were allowed to evaporate at room temperature (32-35˚C) and collected in different Eppendorf tubes. **Buffer-treated protein precipitate was sent to TAAB Biostudy Services, Kolkata, West Bengal, for amino acids quantification and qualification studies. Their results are shown in the results, and the petroleum ether crude extracts were sent to CSIR-IICB,** Kolkata, West Bengal, f**or CHN analysis, and the results are shown.**

6. Determination of the protein concentration of pineapple enzyme

The protein sample was mixed with 10%,20%, 30% ammonium sulfate and centrifuged in 10000 RPM

for 10 min in a cooling centrifuge and precipitate the protein. Then the protein was estimated through the **Lowry method,** and the concentration of protein.

Lowry protein estimation method:

For the determination of protein concentration, we have to prepare

Solution A-2%(w/v) sodium carbonate in 0.1 sodium hydroxide, Solution B-1%(w/v) copper sulphate, Solution C-2%(w/v) sodium potassium tartrate,Solution D-Mix 0.5 volume of solution B , 0.5 volume of solution C and 50 volumes of Solution A, Solution E-Folin Ciocalteau reagent is diluted to 1M acid according to the supplier's instruction.

Procedure

To 1 ml of the test solution, add 5ml of the solution D(Copper reagent), mix thoroughly by vortexing and stand at room temperature for 10min.Add 0.5ml of solution E(Folin Ciocalteau reagent), mix rapidly and incubate for 30,minat room temperature.Measure the absorbance at 600nm against reagent blank not containing protein.The concentration is estimated by preferring to a s standard curve obtained at the same time using known concentration of bovine serum albumin.

Results

1.Identification of different amino acids through LC-MS study

We have received the peaks and data for different amino acids TAAB bioequivalence study center , Jadavpur, Kolkata. The peaks and data are followed.

Table 2: Name, molecular weight and m/z ratio of isolated amino acid

S. no.	Name of amino acid	Molecular weight	Mode of ionization	m/z of amino acid
1	**Glycine**	75.07	Negative	**74.6**
2	**Alanine**	89.09	Negative	**88.7**
3	**Tyrosine**	181.19	Positive	**182.3**
4	**Phenyalanine**	165.19	Negative	**164.2**
5	**Glutamic Acid**	147.13	Negative	**146.6**
6	**Glutamine**	145.15	Positive	**146.4**
7	**Arginine**	174.30	Positive	**175.5**
8	**Histidine**	155.16	Negative	**154.6**
9	**Proline**	200.30	Positive	**201.4**

The water extracts were heated in a water bath, maintaining a temperature up to 30˚C for 6-8 hours. Then, a solid material will appear on the Petri dishes, which are shown in the results. Ethanol extracts are not evaporated for solidification. **Water extracts were then sent to CSIR-IICB, Salt Lake.**

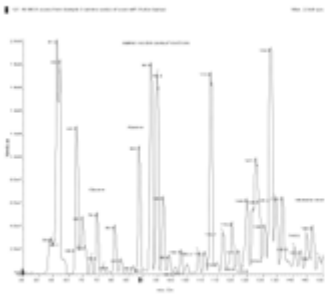


Fig 1: Peaks of Glycine, Alanine, Glutamic acid

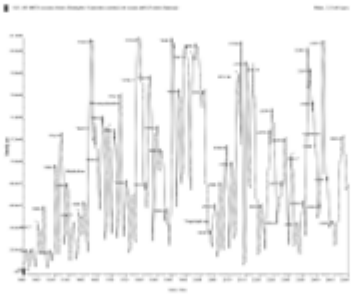


Fig 2 : Peaks of Histidine, Tryptophan, Phenylalanine

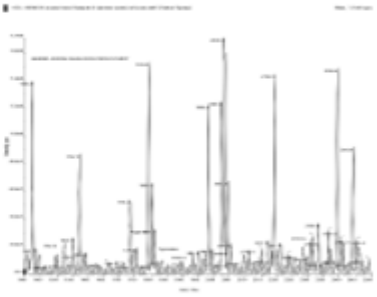


Fig 3: Peaks of Arginine, Tyrosine, Proline

Fig: Peak of different amino acids

Different amino acid which we derived from isolated enzyme of pineapple fruit

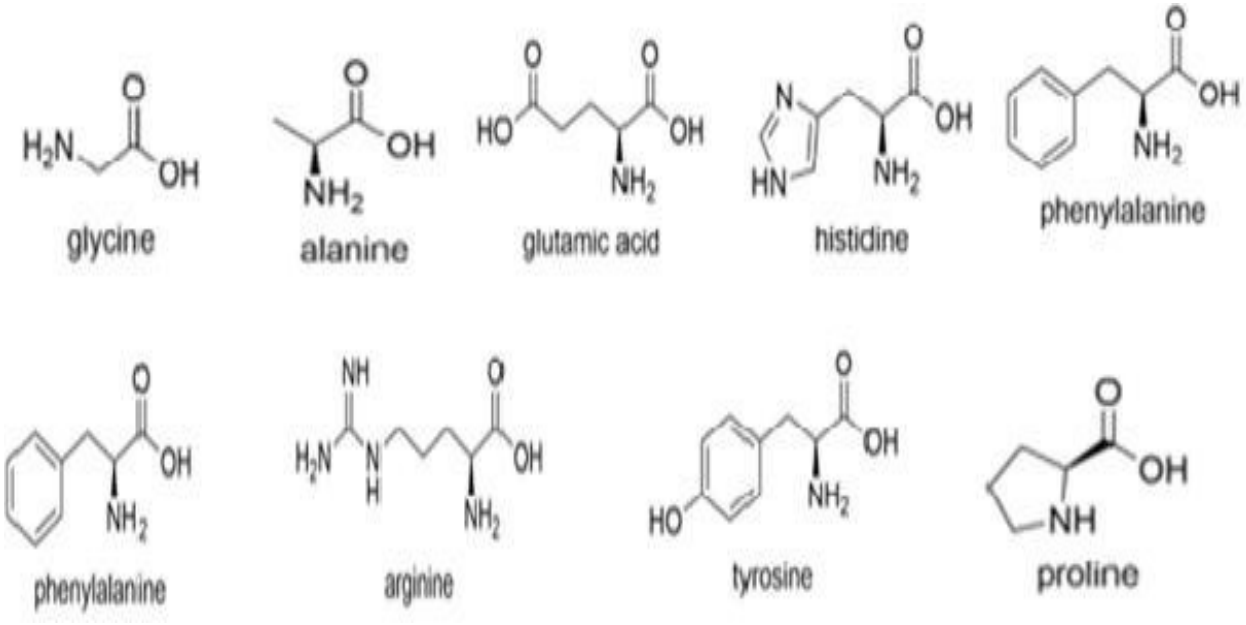


Fig 4: Structure of different amino acid

2.Identification of different phytoconstituents of aqueous extract of *Ananas comosus* through LCMS

Ananas comosus aq extract has been sent to LCMS analysis to IICB Salt Lake campus and different phytoconstituents were identified.**campus, for LC-MS study for different phytochemical analysis,** and **ethanol extracts were sent to CSIR-IICB, Jabalpur campus, for GC-MS** for different phytochemical analysis.

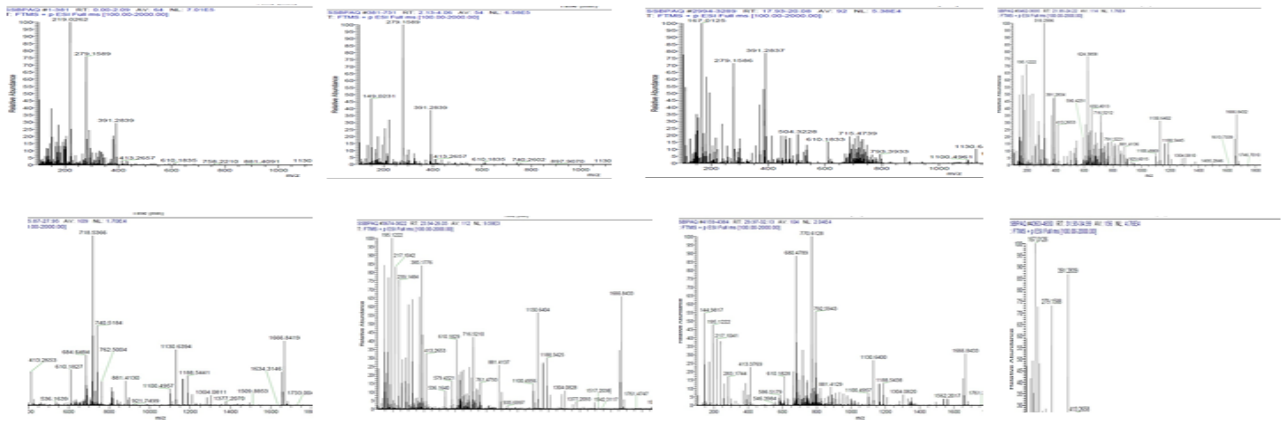


Fig 5: Different peaks of different phytoconstituents of aq extract of pineapple juice

3. Concentration of protein present in pineapple juice determined by Lowry method

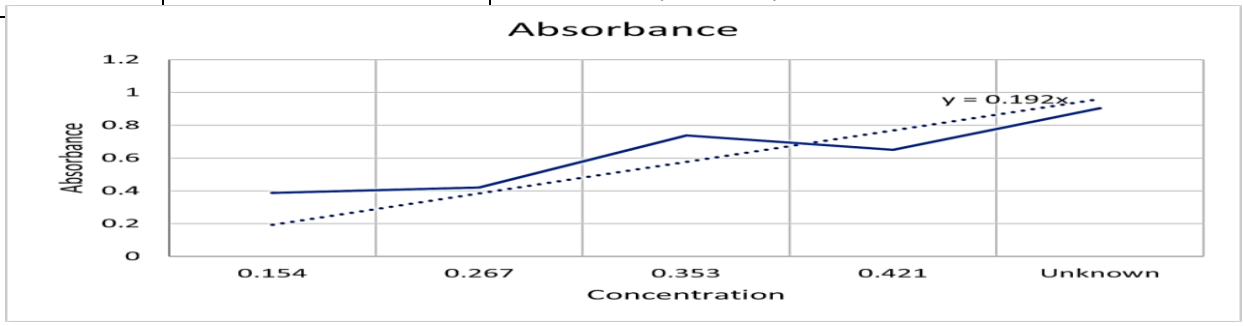
Table 3: Absorbance of the different protein spectroscopy

Sample	Absorbance
1	0.388
2	0.421
3	0.738
4	0.650
5(unknown)	0.903

Table 4: Concentration of the protein sample after measuring in UV-VIS sample by using Lowry method

Protein volume(ml)	Solution D volume(ml)	Folin Catechu reagent volume(ml)	Concentration(ml/ml)
1	5	0.5	0.154
2	5	0.5	0.267
3	5	0.5	0.353
4	5	0.5	0.421

Fig 6: Absorbance curve of the protein sample



So the unknown concentration, x=y/0.192=0.903/0.192=4.7ml per 10.2ml=0.461ml/ml

Table 5: Compound Identified through LCMS

Serial No.	**Compunds name**	**Molecular weight(g/mol)**	Molecular Formula
1	Gallic acid	170.12	**C7H6O5**
2	Pyrocatechol	110.11	**C6H6O2**
3	Hydroxytyrosol	154.16	**C8H10O3**
4	Cholorgenic acid	354.31	**C16H18O9**
5	Caffeic acid	180.16	**C9H8O4**
6	Syringic acid	198.17	**C9H10O5**

7	Trans- 4- coumaric acid	164.16	C9H8O3
8	Ferulic acid	194.18	C10H10O4
9	Ellagic acid	302.19	C14H6O8
10	Myricetin	318.23	C15H10O8
11	Cinnamic acid	148.16	C9H8O2
12	para hydroxybenzoic acid	138.12	C7H6O3
13	Quercetin	302.23	C15H10O7
14	Crytpochlorogenic acid	354.31	C16H18O9
15	Gentisic acid	154.12	C7H6O4
16	Kaemferol	286.24	C15H10O6
17	Apigenin	270.24	C15H10O5
18	Bromelain	1026.9	C39H66N2O29
19	Glucose	180.16	C6H12O6
20	Sucrose	342.3	C12H22O11
21	Galactose	180.16	C6H12O6
22	Maltose	342.3	C12H22O11
23	Fructose	180.16	C6H12O6
24	2- Methyl-3-buten-2-ol	86.13	C5H10O

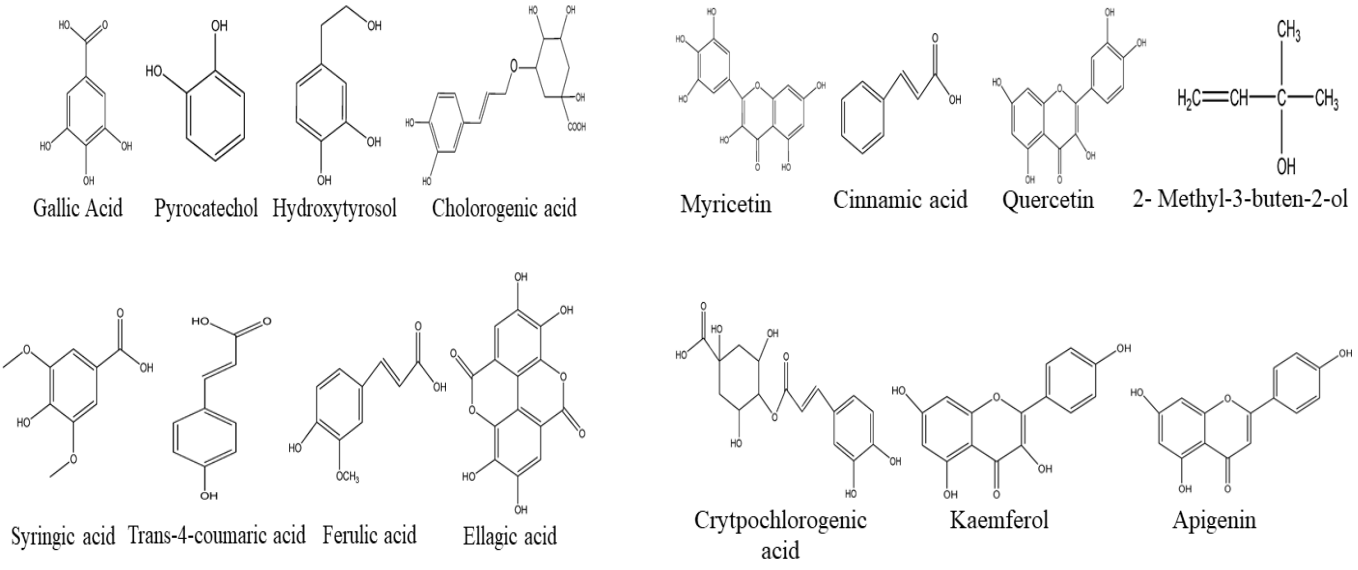


Fig 7: Structure of different phytoconstituents

FTIR DATA INTERPRETITION

The ethanolic and ethanolic-methanolic extract have been anysed under FTIR(Bruker) and the different spectra have been identified.

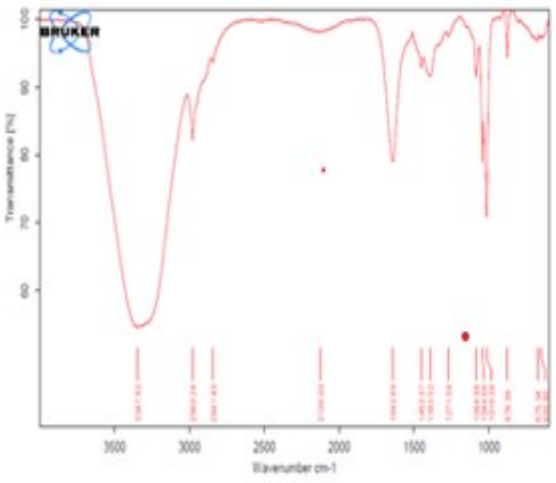


Fig : FTIR spectrum of ethanolic extract of pineapple juice

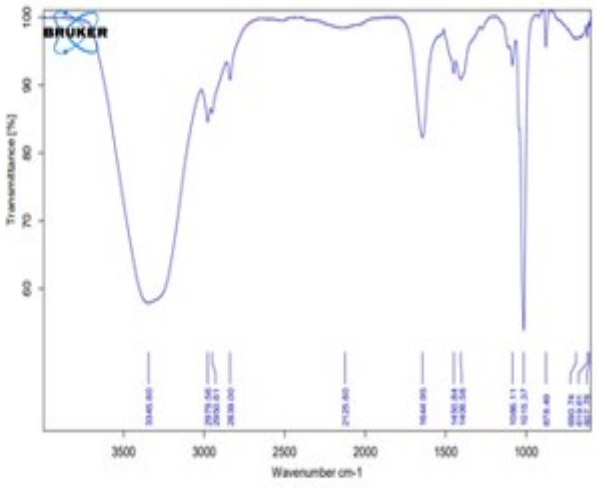


Fig :Ethanolic and methanolic extract of Pineapple juice.

Fig 8: Different IR spectrum of ethanolic and ethanolic-methanolic extract of pineapple juice

The ethanolic data of FTIR shows the peak at 3347nm and shows the presence of Hydroxyl compound, Peak at 1642nm shows the presence of a Diketone compound, peak at 1016nm show the presence of C-O-C group and the peak at 878 nm show the presence of NH wag stretching vibration.

The ethanolic methanolic data of spectrum represents the following compound like the 3345nm peak shows presence of N-H Stretching vibration, the 1645 nm peak shows the presence of Amide (C=O) stretching vibration, 1450-1406 nm peak shows the presence of Alkanes and nitrosoamine,1015 sm peak shows the presence of alkyl amine and peak at 878 nm shows the presence of aromatic compound.

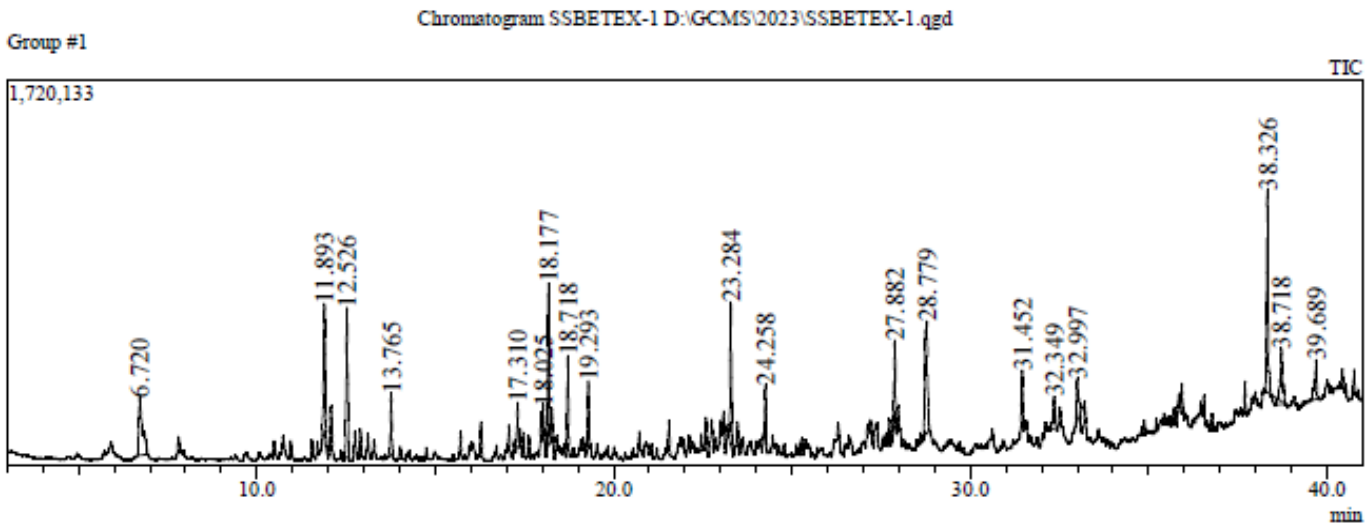
Table 6: Identification of functional group present in Pineapple juice through FTIR

Sl no	Wavenumber	Functional group
1	3347nm	Hydroxyl grp
2	1642nm	Diketone
3	1016nm	C-O-C
4	878 nm	NH wag
5	3345nm	N-H
6	1645 nm	Amide (C=O)
7	1450-1406 nm	Alkanes and nitrosoamine

The ethanolic methanolic data of spectrum represents the following compound like the 3345nm peak shows presence of N-H Stretching vibration, the 1645 nm peak shows the presence of Amide (C=O) stretching vibration, 1450-1406 nm peak shows the presence of Alkanes and nitrosoamine 1015 sm peak shows the presence of alkyl amine and peak at 878 nm shows the presence of aromatic compound.

` **GCMS PEAKS AND DATA DERIVED FROM IICB KOLKATA**

Fig 9: Different phytoconstituents of pineapple juice derived from GCMS



Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%	Name
1	6.720	1838746	7.99	255938	3.51	Decane, 3,7-dimethyl-
2	11.893	2161109	9.39	602033	8.26	Benzene, 1,3-bis(1,1-dimethylethyl)-
3	12.526	1933917	8.40	666697	9.15	Dodecane, 4-methyl-
4	13.765	725441	3.15	274342	3.76	Dodecane, 2,6,11-trimethyl-
5	17.310	453019	1.97	197294	2.71	HEPTADECANE
6	18.025	204092	0.89	153894	2.11	2,6,10-Trimethyltridecane
7	18.177	1798080	7.81	719132	9.86	NONADECANE
8	18.718	1154100	5.02	444433	6.10	2,4-DITERT-BUTYLPHENOL
9	19.293	672147	2.92	282016	3.87	NONADECANE
10	23.284	1632154	7.09	632701	8.68	NONADECANE
11	24.258	616750	2.68	265282	3.64	Hexadecane
12	27.882	981481	4.27	385143	5.28	Eicosane
13	28.779	2507185	10.90	495175	6.79	HEXADECANOIC ACID
14	31.452	706211	3.07	259684	3.56	11,14-EICOSADIENOIC ACID, METHYL I
15	32.349	572992	2.49	145670	2.00	Eicosane
16	32.997	66770	0.29	190034	2.61	OCTADECANOIC ACID
17	38.326	3143969	13.66	897725	12.31	1,2-BENZENEDICARBOXYLIC ACID
18	38.718	1172790	5.10	248851	3.41	Heneicosane
19	39.689	670562	2.91	174112	2.39	DOCOSANE
		23011515	100.00	7290156	100.00	

CONCLUSION

Throughout the experimental work we have found the pineapple juice contain different amino acid like glycine, proline, glutamic acid, histidine, arginine etc. Through LCMS we have received Gallic acid, Pyrocatechol, Hydroxytyrosol, Chlorogenic acid, Caffeic acid, Syringic acid, Trans-4-coumaric acid, Ferulic acid, Ellagic acid, Myricetin, Cinnamic acid, para-hydroxybenzoic acid, Quercetin, Cryptochlorogenic acid, Gentisic acid, Kaemferol, Apigenin, Bromelain, Glucose, Sucrose.

The compounds identified in the GCMS are decane, 3,7-dimethyl-; benzene, 1,3-bis(1,1-dimethylethyl)- dodecane, 4-methyl-; dodecane, 2,6,11-trimethyl-; heptadecane; 2,11-2,6,10-trimethyltridecane; nonadecane; 2,4-ditertiarybutylphenol; nonadecane; hexadecane; eicosane; hexadecanoic acid; 11,14-eicosadienoic acid, methyl; eicosane; octadecanoic acid; 1,2-benzenedicarboxylic acid; heneicosane; and docosane.

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Conflict of interest

Any of the authors do not have any conflict of interest with any financial authority. There is no conflict of interest in which professional judgment concerning a primary interest (such as a patient's welfare or the validity of research) tends to be unduly influenced by a secondary interest (such as financial gain).

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