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Biocharacterization Study on Fermented Liquid Organic Manure (*Kunapajala*) Using Analytical Technique: Gas Chromatography – Mass Spectrometry (GC-MS)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

"Vrikshayurveda", an ancient literature collection of plant life science depicted importance of *kunapajala*, a fermented liquid organic manure sourced from animal origin. It is an excellent organic fertilizer influencing a wide range of crops with enhanced growth and development. During later days, "*kunapajala*" with vegetarian ingredients, without nutrient compromise was developed and

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standardized. It includes ingredients *viz.,* soybean meal, paneer, tofu, rice husk, black gram, groundnut oilcake, cow dung, urine, honey, ghee and milk. The fermented formulation has essential nutrients, amino acids, keratins etc. which applied on plants, naturally flourished with excellent growth, flowering and fruiting. In this study, the fermented liquid organic manure *kunapajala* at 20th, 40th and 60th day of fermentation period was analysed for its bioactive compounds using the analytical technique Gas Chromatography – Mass Spectrometry (GC-MS). Extract was prepared using organic solvents diethyl ether and methanol. The mass spectra of compounds matched with Wiley's standard mass spectrum database and National Institute of Standards and Technology's (NIST) libraries. This study unfolded the presence of various compounds like Hexanoic acid, Phenol-TMS, Methyl oleate, Octadecanoic acid, Methyl palmitate, Myristic acid-TMS, Tetradecanoic acid, Caproic acid, Methyl myristoleate; (Z)-tetradec-9-enoic acid, 2-Aminooctanoic acid-TMS; 2-aminooctanoic acid, Butylated Hydroxytoluene, Juniperic acid-2TMS, Stearic acid-TMS, Linoleic acid-TMS etc. These phytochemicals have antimicrobial, antifungal, antibacterial, physiological and metabolic responses, antioxidant, Insecticidal irritant and nematicidal activities which effectively influence organic crop production. The result indicated the vital role of *kunapajala* as an effective source of liquid formulation through its phytochemical importance.

Keywords: Vrikshayurveda; organic liquid formulation; kunapajala; diethyl ether; GC-MS; phytochemicals.

1. INTRODUCTION

The present global context emphasizes significance of implementing eco-friendly farming practices for agricultural sustainability over long run. Persistent use of chemical inputs in agriculture, possesses serious consequences on diverse ecosystems particularly after green revolution era and this issue has profound effect in food grain production. Thus, demanding for organic produces and their consumption has been drastically increased among today's health-conscious society. Hence, farmers all over the world are making attempts to detoxify the system by switching to organic farming for the betterment of future world. Organic agriculture is practiced in 187 countries in an area of 72.3 m ha managed organically by 3.1 million farmers. Australia has greatest organic agricultural land (35.69 m ha), followed by Argentina (3.63 m ha) and Spain (2.35 m ha). In India, total organic cultivation area is 2.30 million acres and accounts for 30% of the world's total organic cultivation and has 27,59,660 farmers, 1703 processors, and 745 dealers. The cultivation of plants using organic preparations dates back to 1000 AD in India. *'Vrikshayurveda'* by *Surapala* is a documentation of ancient science of plant life that deals with the cultivation of numerous plant species, their healthy growth, and productivity and the usage of fermented liquid organic manure known as *'kunapajala'* for plant sustenance and was published around 1000 AD [1]. Details about *'Kunapajala'* can also be found in *'Upavanavinoda'*, an anthropological compilation called *'Sharangadhara Paddhati'* compiled in 13th century by *Acharya Sharangadhara*. "*Kunapa"* means "smelling like a dead body or stinking" in Sanskrit. *kunapambu* or *kunapajala* was suitably named since it entailed the fermentation of animal remnants, such as meat and marrow, with a foul odour [2]. The fermented liquid organic manure was developed using animal wastes that include animal flesh, bone marrow, skin, dung and urine. Plants responded very well to the nourishment provided by *kunapajala* and flourished with excellent growth, flowering, and fruiting, because the fermented product contained basic constituents such as amino acids, sugars, fatty acids, keratins, macro- and micronutrients in available form [3]. Essential amino acids *viz.,* arginine, isoleucine, valine methionine and so on were available in the traditionally fermented liquid manure *kunapajala* in which amino acid like mathionine act as precursor of plant growth regulators [4]. Application of *kunapajala* on 10th and 15th days resulted in significant increase in paddy growth [5]. It has been observed that application of *kunapajala* on a daily basis improves soil fertility and plant growth and use of *dhanyagavya*, a tea-leaf pesticide made from cow dung, water and rice husk, aided in the eradication of the insect [6]. A study on langali (*Gloriosa superba* Linn.) indicated that using modified *kunapajala* surpassed the control and the group cultivated according to modern agricultural standards in terms of yield [7]. The combined effect of *kunapajala* and *panchgavya* on chilli, cowpea and tomato seedlings resulted in an induced defense mechanism in the plant body which in turn reduces the disease incidence and promoted the growth and yield attributes of the crops [8]. GCMS can separate and quantify multi-component samples and complicated matrices, as well as identify unknown substances. It is frequently used for direct separation and analysis of air samples. Hence, the current study was designed to examine the phytochemical compounds and its biological influence on plants that is present in the fermented liquid organic manure (*kunapajala*) at 20th, 40th and 60th days of fermentation period through analytical technique involving Gas Chromatography – Mass Spectrometry and the results are discussed in this paper.

2. MATERIALS AND METHODS

2.1 Preparation of Fermented Liquid Organic Manure (Modified *kunapajala* with Vegetarian Base)

The fermented liquid organic manure (modified *kunapajala* with vegetarian base) was prepared in Department of Sustainable Organic Agriculture, Agricultural College and Research Institute, Tamil Nadu Agricultural University (TNAU), Coimbatore. The technique for preparing modified *kunapajala* with vegetarian base using the ingredients listed in Table 1 [3]. The ingredients Soybean meal + Paneer (100 g + 100 g), Tofu from soybean (100 g), Rice husk (100 g), Black gram (50 g), Groundnut oilcake (100 g) has to be cooked with 1 – 1.5 litre or more water for about 30 minutes and allow it to cool. Then, transfer the contents to a 50 litre non corrosive plastic drum. Then add cow dung (1 kg), cow urine (1 l), honey (25 g), ghee (25 g), milk (100 ml) to the drum and add water to make the total capacity to 20 litres. The mixture has to be stirred twice daily in both clockwise and anticlockwise gently for a period of required duration (20 days, 40 days and 60 days of fermentation). The fermented liquid manure modified *kunapajala* was then filtered by using cotton cloth and the filtrate collected was subjected to further analysis. The sample for analysis were collected at 20, 40 and 60 days after fermentation. The biochemical characteristics of modified *kunapajala* prepared using this method were determined using GC-MS.

2.2 Preparation of Extract for GC-MS Analysis

The fermented liquid organic manure (*kunapajala*) was collected freshly at 20-, 40- and 60-days duration of fermentation period and the filtrate was again filtered through Whatman no.1 filter paper for biocharacterization study in Gas Chromatography – Mass Spectrometry (GC-MS). Take 20 ml of *kunapajala* filtrate in 500 ml separation funnel. Add 100 ml of diethyl ether solvent (25 ml each for four times) and swirl gently for 5-10 minutes each. The separation funnel was allowed to stand for 10-15 minutes until the pressure diminished and the formation of two distinct layer (‘colourless top organic phase’ and ‘bottom aqueous phase’). The sample was extracted with diethyl ether organic solvent. The extraction in solvent was carried out until the additional solvent became colourless. Now, the organic solvent was collected in the conical flask and aqueous layer was discarded. The organic layer was decanted into a flask by passing it through sodium sulphate (Na_2SO_4). 50 ml of diethyl ether was added and thoroughly shaken and the pressure was released intermittently. It was again permitted for 10 to 15 minutes. The filtrate was concentrated to dryness using a Rotary vacuum evaporator, followed by addition of 5 ml of methanol and dried again. Finally, 1 ml of methanol (HPLC grade) was added and transferred to a vial for GC-MS analysis.

Table 1. Ingredients for preparation of modified *kunapajala* with vegetarian base

S. No.	Ingredients	Quantity
1.	Soybean meal + Paneer	100 g + 100 g
2.	Tofu from soybean	100 g
3.	Rice husk	100 g
4.	Black gram	50 g
5.	Groundnut oilcake	100 g
6.	Cow dung	1 kg
7.	Cow urine	1.5 l
8.	Honey	25 g
9.	Ghee	25 g
10.	Milk	100 ml

2.3 Gas Chromatography – Mass Spectrometry (GC-MS) Analysis

GC-MS is an analytical technique that combines gas chromatography (GC) and mass spectrometry to identify the unknown compounds or substances. The detection technique of Mass Spectrometry (MS) generates, separates, and detects ions in the gaseous phase. When connected to a GC, it ionizes the gaseous eluted substances immediately, separates the ions in vacuum based on their mass-to-charge ratios (m/z), and finally measures the intensity of each ion. These intensities are recorded in order to generate a series of mass spectra that show the relative ion intensities against m/z. The mass chromatogram is the final result of GC-MS. GCMS separates and quantifies multi-component samples and complicated matrices, as well as identifying unknown substances. The extract of fermented liquid organic manure (*kunapajala*) was analyzed using Shimadzu smart technologies GCMS-TQ8040 NX type. An injection volume of 20 µL was employed for the analysis. The instrument linked with SH-Rxi-5Sil MS column (30.0 m Column Length, 0.25 mm Inner Diameter, 0.25 µm Film Thickness). The column oven temperature and equilibration time was kept at 70.0°C and 1.0 minute respectively. With GCMS-TQ series, Ion Source Temperature (230°C), Interface Temperature (280°C) and Solvent Detector Voltage of 0.1 kV was configured along with Start Time (5.00 min), End time (55.00 min) and Event Time (0.100 sec). As a carrier gas, helium was used at a flow rate of 1.0 mL/minute. The GC-MS instrument were employed with the above conditions and analysis of samples were done.

2.4 Identification of Chemical Compounds

The relative quantity of chemical components present in the extract was expressed as a percentage based on peak area produced in the chromatogram. The identification was validated using the molecular structure, molecular mass, and calculated fragments. Wiley's standard mass spectrum database and the National Institute of Standards and Technology's (NIST) libraries were used to analyse the GC-MS data. The collection contains almost 1 million EI (Electron Ionisation) mass spectra, 973,000 chemical structures, and 770,000 distinct molecules. The NIST possesses approximately 306,622 EI mass spectra with a total of 267,376 unique constituents. WILEY 8 and FAME have approximately 65,000 designs [9]. The phytochemical activities listed in Tables 2, 3 and 4 are based on Dr. Dukes's Phytochemical and Ethonobotanical Database by Dr. Jim Duke of the Agricultural Research Services/USDA [10].

2.5 Profiling of Phyto-Chemical Compounds

The presence of *kunapajala's* phytochemical composition was determined using GC-MS. The phytochemicals were identified using the Wiley and NIST libraries based on their Retention Time (RT), Peak Area Percentage (%), Height (%), A/H ratio, Molecular Formula, and Molecular Mass. The GC-MS chromatogram from *Kunapajala* at 20th, 40th and 60th days of fermentation revealed different peaks has projected in figure as Fig. 1, Fig. 2 and Fig. 3, respectively.

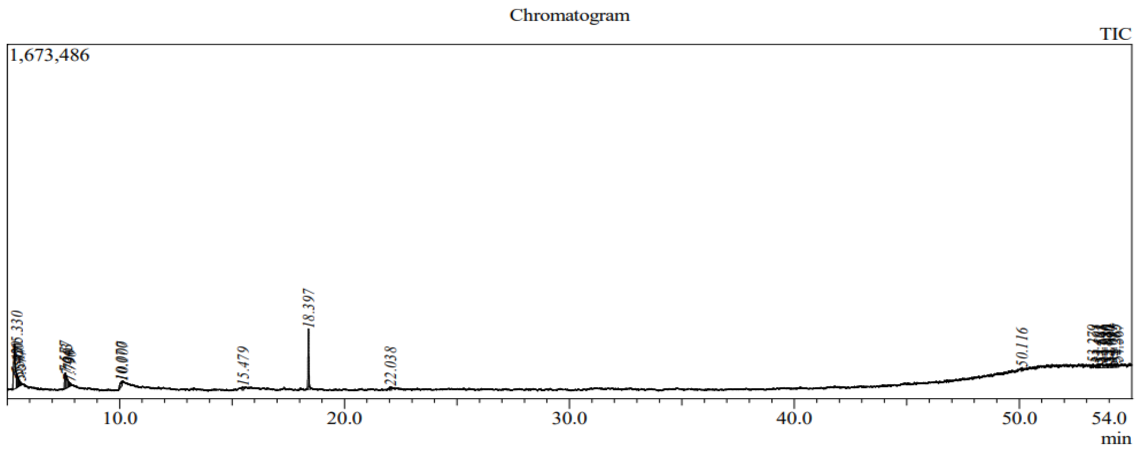

Fig. 1. GC-MS Chromatogram of *kunapajala* extract at 20th day of fermentation period

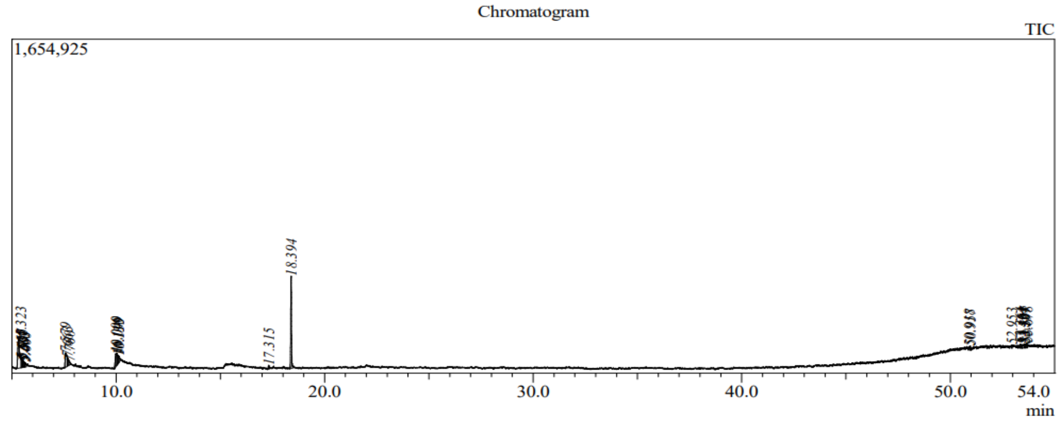

Fig. 2. GC-MS Chromatogram of *kunapajala* extract at 40th day of fermentation period

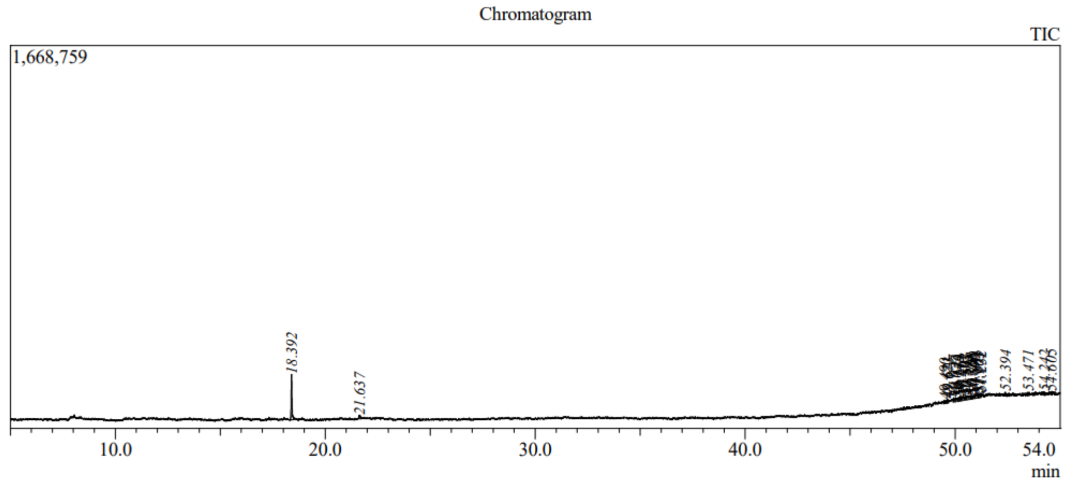

Fig. 3. GC-MS Chromatogram of *kunapajala* extract at 60th day of fermentation period

3. RESULTS AND DISCUSSION

GC-MS was employed to perform specific tests and has been universally hailed as "gold standard" for forensic substance identification and has identified presence of actual compounds. In present study, bio-characterization of fermented liquid organic manure *kunapajala* was done to identify the biologically active compounds present in liquid formulation using the analytical technique Gas Chromatography – Mass Spectrometry. The samples were collected at 20th, 40th, and 60th day of fermentation period. Different compounds were identified using GC-MS with their Retention Time (RT), Peak Area (%), Height (%), A/H, Molecular Formula, Molecular Weight and its biological activities of the phytochemicals during different fermentation periods of 20th, 40th and 60th days is tabulated in Table 2, Table 3 and Table 4, respectively.

The GC-MS analysis of *kunapajala* unfolded various alkanes, alkanols, alcohols, fatty acids and phthalate phytocompounds. At 20th day, it recorded predominant peak area of 27.49 % of hexanoic acid, which is found to have an antifungal property [11], followed by Phenol-TMS, Methyl oleate, octadecanoic acid (5.72%), Tetradecanoic acid (2.89%) and so on. During 40th day of fermentation, compounds like Hexanoic acid, Caproic acid, n-Caproic acid (22.79%) recorded the highest peak area followed by Butylated Hydroxytoluene (27.24%) and so on. These compounds have the properties as preservative in foods, animal feed, additive, antifungal and antimicrobial activity. With 60th day of fermentation period, Butylated Hydroxytoluene or Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl recorded the highest peak area of 37.02% and the compound is found to have antioxidant, antifungal property, used as preservative in vegetable oils, petroleum

Table 2. GC-MS analysis report of *kunapajala* at 20th day of fermentation period

S. No.	Retention Time (RT)	Peak Area (%)	Height (%)	A/H	Compound Name	Molecular Formula	Molecular Weight	Phytochemical Activity
1.	5.330	27.49	19.23	5.14	Hexanoic acid	$C_6H_{12}O_2$	116	Induced resistance against *Botrytis cinerea,* Antifungal activity [11]
2.	7.643	5.72	5.04	4.08	Phenol-TMS, Methyl oleate, Octadecanoic acid	$C_{19}H_{36}O_2$	296	Antibacterial, Antifungal, insect antifertility and antimicrobial activity [15]
3.	50.116	1.20	1.44	3.00	3-Hydroxyvaleric acid-2TMS, Linoleic acid-TMS	$C_{21}H_{40}O_2$	352	Insecticidal and nematocidal activity [16]
4.	53.279	0.60	1.71	1.26	Urocanic acid- 2TMS or Benzoic acid	$C_{13}H_{22}O_3$	282	Increased Aromatic Amino Decarboxylase Activity, Production of Uric Acid inhibitor activity [17]
5.	53.940	0.86	1.44	2.14	Methyl tridecanoate or Methyl palmitate or Hexadecanoic acid	$C_{17}H_{34}O_2$	270	Antibacterial, Antifungal and Pesticidal activity [15]
6.	54.088	1.18	1.44	2.83	Myristic acid-TMS	$C_{17}H_{36}O_2$	300	Larvicidal, Antioxidant and repellent activity [18]
7.	54.090	2.89	1.40	3.09	Tetradecanoic acid	$C_{17}H_{36}O_2$	300	Insect Repellant and Antioxidant activities [15]
8.	54.091	1.25	1.45	3.09	Octadecanoic acid	$C_{21}H_{44}O_2$	356	Antifungal, Antimicrobial and insect antifertility activity [15]
9.	54.367	2.02	1.60	4.52	Methyl cis-13, 16-Docosadienate or Oleic acid	$C_{21}H_{42}O_2$	354	Insecticidal irritant, Antibacterial activity [16]
10.	54.367	2.03	1.62	4.52	Methyl linolelaidate	$C_{19}H_{34}O_2$	294	Larvicidal and antimicrobial activity [19]

Table 3. GC-MS analysis report of *kunapajala* at 40th day of fermentation period

S. No.	Retention Time (RT)	Peak Area (%)	Height (%)	A/H	Compound Name	Molecular Formula	Molecular Weight	Phytochemical Activity
1.	5.323	22.79	11.82	6.20	Hexanoic acid, Caproic acid, n-Caproic acid	$C_6H_{12}O_2$	116	Antifungal and Antimicrobial activity [11]
2.	5.537	1.20	2.65	1.46	Methyl oleate, (Z)-octadec-9-enoic acid	$C_{19}H_{36}O_2$	296	Antibacterial, Antifungal and Antimicrobial activity [15]
3.	5.660	1.28	1.44	2.88	Methyl myristoleate; (Z)-tetradec-9-enoic acid	$C_{15}H_{28}O_2$	240	Insect repellant and larvicidal, Antioxidant activity [15]
4.	7.663	3.89	3.67	3.40	2-Aminooctanoic acid-TMS; 2-aminooctanoic acid	$C_{11}H_{25}NO_2$	231	Antimicrobial, Increased Aromatic Amino Acid Decarboxylase Activity and have Insecticidal property [17]
5.	10.00	7.21	4.86	4.77	Octanoic acid, n-Caprylic acid, n-Octanoic acid, n-Octoic acid	$C_8H_{16}O_2$	144	Insecticidal and antimicrobial activity [18]
6.	18.394	27.24	34.69	2.53	Butylated Hydroxytoluene	$C_{15}H_{24}O$	220	Preservative in foods, animal feed, additive, antifungal and antibacterial activity [12]
7.	52.953	0.53	1.21	1.39	Protocatechuic acid-3 TMS; 3,4-dihydroxybenzoic acid	$C_{16}H_{30}O_4$	370	Arachidonic acid inhibitor, and Increased Aromatic Amino Acid Decarboxylase Activity [17]
8.	53.451	0.71	1.27	1.81	Succinylacetone-meto-TMS (4), Oleic acid- TMS	$C_{21}H_{42}O_2$	354	Antibacterial and Anemiagenic Insecticidal activity [16]
9.	53.567	0.45	1.55	0.93	Juniperic acid-2TMS, 16-hydroxyhexadecanoic acid,	$C_{22}H_{48}O_3$	416	Antifungal, Pesticidal and Antibacterial activity [15]
10.	53.597	0.85	1.48	1.84	Dodecanedioic acid-2TMS, dodecanedioic acid	$C_{18}H_{38}O_4$	374	Antimicrobial activity [15]

Table 4. GC-MS analysis report of *kunapajala* at 60th day of fermentation period

S. No.	Retention Time (RT)	Peak Area (%)	Height (%)	A/H	Compound Name	Molecular Formula	Molecular Weight	Phytochemical Activity
1.	18.392	37.02	34.62	2.87	Butylated Hydroxytoluene or Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl	$C_{15}H_{24}O$	220	Antioxidant, used as preservative in vegetable oils, petroleum products, and Antifungal activity [20]
2.	50.179	2.62	2.84	2.48	Methyl pentadecanoate; Pentadecanoic acid	$C_{16}H_{32}O_2$	256	Lubricant and Antioxidant activity [21]
3.	50.392	5.94	2.93	5.44	4-Aminobenzoic acid-2TMS; 4-aminobenzoic acid	$C_{13}H_{23}NO_2$	281	Uric acid production and Arachidonic acid inhibitor [17]
4.	50.595	3.91	2.77	3.78	Stearic acid-TMS; octadecanoic acid	$C_{21}H_{44}O_2$	356	Antimicrobial, antifungal and Antibacterial activity [15]
5.	50.766	1.75	2.59	1.80	Linoleic acid-TMS; octadeca-9,12-dienoic acid	$C_{21}H_{40}O_2$	352	Insect antifertility, Antibacterial and Antifungal activity [18]
6.	50.808	1.29	2.52	1.37	7-Hydroxoctanoic acid-2TMS; 7-hydroxyoctanoic acid	$C_{14}H_{32}O_3$	304	Antimicrobial and Insecticidal activity [22]
7.	51.103	1.13	2.58	1.17	Juniperic acid-2TMS; 16-hydroxy hexadecanoic acid	$C_{22}H_{48}O_3$	416	5-Alpha-reductase inhibitor, Antioxidant, Antifibrinolytic, Lubricant, Pesticide, Nematicidal activity [21]
8.	51.171	2.76	2.63	2.81	Methyl myristoleate; (Z)-tetradec-9-enoic acid	$C_{15}H_{28}O_2$	240	Insect repellent and Antioxidant property [18]
9.	52.394	1.05	2.75	1.02	Arachidonic acid-TMS; (5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid	$C_{23}H_{40}O_2$	376	Enzymatic activity, Lipoxygenase and Cytochromes P-450 substrate [23]
10.	54.242	2.35	2.44	2.58	Succinylacetone-meto-TMS (1); 4,6-dioxoheptanoic acid	$C_{12}H_{24}N_2O_4$	288	Inhibit ALA dehydratase enzyme, Inhibit ^{14}C amino acid uptake [24]

products [12]. The presence of bioactive compounds such as methyl palmitate, gallic acid, methano-bis dasycarpidan-1-methanol, sphinganine methaneboranate and so on were unfolded in the study on panchagavya shelf-life analysis with different alternatives for ghee using GC-MS [13]. Potential bioactive fractions *viz.,* 1-Heneicosanol, n-Heptadecanol-1, n-Nonadecenol-1, 6H-pyrazolo [1,2-a] [1,2,4,5] tetrazine, 2-pentanone, 4-hydroxy-4-methyl etc., were identified from GC-MS analysis of cow urine which exhibited significant role in biological activities [14]. The GC-MS analysis of fermented liquid organic formulation *kunapajala* at 20^{th}, 40^{th} and 60^{th} days of fermentation period expressed a variety of phytochemical compounds such as Myristic acid-TMS, Methyl palmitate, Oleic acid, Methyl linolelaidate, Protocatechuic acid-3 TMS, Juniperic acid-2TMS, Dodecanedioic acid-2TMS, Stearic acid-TMS, Octadecanoic acid, Arachidonic acid-TMS, 4-Aminobenzoic acid-2TMS and so on with the highest retention time tend to possess various biological activities such as antifungal, antibacterial, antimicrobial, antioxidant, nematicidal, pesticidal, insecticidal irritant, herbicidal activities, etc.

4. CONCLUSION

Kunapajala, a fermented liquid organic manure gaining its popularity because to its significant impact on a wide range of crops. The usage of organic liquid nutrition solutions resulted in increased crop growth and development, resistance to insect pest and diseases and improves yield and quality of the crop. This increases interest in using liquid formulations in organic farming and is based on a system-oriented approach and ensures environment sustainability. Hence, this fermented liquid organic manure *kunapajala* has been subjected for biological characterization study using the analytical technique Gas Chromatography – Mass Spectrometry (GC-MS). The result shows the presence of various phytochemical compounds. These compounds play a vital role in antifungal, antimicrobial, insect repellent, insect antifertility, antioxidant and physiological responses. From this study, it can be concluded that this fermented liquid organic manure *kunapajala* may serve as a potential source of liquid nutrient formulations in organic farming due to the presence of various phytochemicals and biologically active compounds which has impact on the crop growth and development. As bio-characterization of *kunapajala* continue to evolve, there are several potential future directions that may be explored *viz.,* identification and quantification of additional compounds, compounds interaction with biological systems, standardization and quality control. Overall, bio-characterization of fermented liquid organic manure can help to ensure its quality and effectiveness in turn improving the sustainability of agricultural production.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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