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Research Article

PHYSICOCHEMICAL ANALYSIS AND CHROMATOGRAPHIC PROFILING USING LC-MS OF *KANJIKA* DURING FERMENTATION

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ABSTRACT

Kanjika is a traditional Ayurvedic fermentation product prepares using 11 herbal ingredients in an aqueous media without the addition of any sweetening agents. The aim of the study was to analyse the biochemical changes happening throughout the process of fermentation in *Kanjika* and to identify the bioactive molecules formed during the fermentation process. Two batches of *Kanjika* were prepared. One batch was kept undisturbed throughout the fermentation process, while samples were drawn out at regular intervals from the other batch. A comparative evaluation of physicochemical parameters was done between the various samples drawn from the second batch of the formulation and the sample from the first batch after completion of fermentation. Further detailed analysis of the product before initiation of fermentation and after completion of fermentation was done using LC-MS. The two batches of *Kanjika* showed significant changes with regard to basic physicochemical parameters. It was found that in both the batches the pH was found to be highly acidic without any significant variations among the samples. Brix value was significantly high at the end of fermentation, in the sample from the undisturbed batch when compared to the sample from the disturbed batch.

INTRODUCTION

Fermented preparations hold a very important role in Ayurveda due to their prolonged shelf life and enhanced therapeutic efficacy. The procedure adopted to prepare fermented preparations in Ayurveda can be termed broadly as *Sandhana Kalpana*. These fermented products are classified into alcoholic preparations and acidic preparations. Fermented beverages are defined as those products that have been subjected to the effect of microorganisms or enzymes to cause desirable biochemical changes[1]. Majority of the traditional Ayurvedic acidic preparations are formed due to cereal fermentation. *Kanjika* is one such unique preparation, which is an acid based, cereal fermented product.

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Cereal fermentation is the lactic acid fermentation of cereals. It is a processing method which has been established for a long time. The presence of lactic acid bacteria in *Kanjika* which qualifies the preparation as a probiotic indicates that the fermentation process involved in the preparation of this formulation is lactic acid fermentation[2]. It has been found that increased levels of free amino acids and their derivatives in fermentation products are involved in cereal fermentation by lactic acid bacteria. The final products might be formed as a result of proteolysis or by metabolic synthesis[3]. It has also been found that fermentation lowers the content of anti-nutrients like phytates, tannins and polyphenols[4].

LAB has been isolated from *Kanjika* which indicates the ongoing process of acidic fermentation in the formulation [2]. LAB helps in the fermentation of glucose into lactic acid and acetic acid along with ethanol and CO_2[5]. Studies have shown that lactic acid fermentation of rice enhance the flavour, nutritive value and available lysine content[6]. Bamboo leaves also affect fermentation characteristics[7]. Bamboo leaves as an initiator in lactic acid fermentation products has been explored in many studies. *Kanjika* is a preparation in which bamboo leaves are made use for initiating fermentation. The interaction of bamboo leaves with the cereals and other ingredients made use in the preparation plays an important role in the incorporation of bioactive molecules that aid in its pharmacological activity.

Kanjika is explained in Ayurvedic classical literature in the context of purification of many of the metals and minerals[8]. It contains Oryza sativa, Sodium chloride, *Vigna mungo, Brassica Nigra, Curcuma longa, Bambusa vulgaris, Cuminum cyminum, Zingiber officianale, Ferula asafoetida* and *Macrotyloma uniflorum.* The process of purification of metals involves quenching of the red-hot metal into Kanjika[8]. After this procedure, we are only able to observe the physical changes happening to the media as well as the metal. The mode of action of this formulation with reference to its interaction with metals in particular has not yet been studied. In order to study in detail about the chemical interactions that are taking place during this process, it is necessary to analyse and confirm the presence of the various bioactive molecules present in this preparation. Till date, only a preliminary analysis of *Kanjika* has been carried out[9]. As a preliminary step towards such an elaborate understanding, the physio chemical parameters of *Kanjika* must be studied for in detail. Also, in order to understand the various interactions that are taking place, identification of the biologically active molecules present in the formulation is necessary. In this paper, an attempt is made to compare and analyse the physico-chemical parameters of disturbed and undisturbed samples of *Kanjika* during the process of fermentation to understand the changes happening during this process. The assessment of bioactive components in the samples during various time intervals during fermentation was also done using LC-MS.

MATERIALS AND METHODS

Preparation of *Kanjika*

Total 2 litres of *Kanjika* was prepared in 2 batches of 1 litre each. Decoction of horse gram (*Kulatha kwatham*) was prepared by taking 100 g horsegram (*Kulatha*) along with 1600 ml of water and was reduced to 200 ml. The container was subjected for fumigation and smeared with a little amount of *Sarshapa taila* (sesame oil). Prepared *Kulattha Kwatha* along with rice was added to it. *Masha vatakas* (pancakes made of black gram flour) prepared and fried in sesame oil were added to it. *Ashuddha hingu* (Asofoetida) was subjected to frying in sesame oil and was added. Powders of the rest of the ingredients were added in prescribed quantity along with cleaned and dried fresh bamboo leaves. 800 ml of water was added to the mixture followed by the addition of sesame oil. The ingredients were mixed thoroughly and the mouth of the container was sealed and kept for fermentation for a period of 30 days. After completion of fermentation, fresh *Kanjika* was collected by filtration. (Figure 1)

Fig. 1: Preparation of *Kanjika*

Sampling for the study

One batch of the formulation was kept undisturbed till the 4th week while samples were drawn weekly from another batch of the formulation. Samples for preliminary analysis of disturbed batch were named as KD_1 (week 1), KD_2 (week 2), KD_3 (week 3) and KD_4 (week 4). Two samples namely K_0 (week 0) and KU_4 (week 4) were drawn from undisturbed batch. Samples for microbial load assessment were named as Sample1_before processing, Sample2_disturbed final and Sample3_undisturbed final. Samples for LC-MS study were named as KANJI I (before processing) and KANJI II (undisturbed sample after 4 weeks).

Comparative physico-chemical analysis of *Kanjika*

Physico-chemical analysis was done to ascertain the quality of the formulation. Determination of organoleptic characteristics viz. odour, taste, colour, pH, Brix and Specific gravity of the prepared formulation of *Kanjika* was carried out. All these parameters were analysed for the formulation before processing, after 4 weeks of fermentation and also of a sample of the formulation that was kept undisturbed for 4 weeks.

LC-MS analysis

The LC-MS analysis was done using Agilent technologies 1260 infinity HPLC instrument and Ms-6120 Quadrupole with Agilent-Eclipse plus C column of dimensions 184.6 ×250 mm. The mobile phase selected was Acetonitrile: 0.1% Formic acid in water in the ratio 60:40. The procedure was carried out with an injection volume of 10µL with a flow rate of 0.4ml/mint. The drying gas flow was 12(1/min) under a Nebulizer pressure of 50 psig, drying gas temperature of 350°C, capillary voltage of 4000 V and positive polarity. The given sample was filtered through syringe filter and injected to the LCMS instrument. The sample was made to run through the instrument for a period of 40 mint. LC-MS study of two samples, KANJI I(sample before processing) and KANJI II(undisturbed sample after 4 weeks of fermentation) were assessed.

RESULTS

Kanjika is a cereal fermentation product prepared using 11 herbal ingredients (Table 1). Temperature and seasonal changes highly affect the rate and quality of fermentation. According to classical literature, the preparation is said to be ready in 7 days. As per previous study reports, fermentation was initiated only on the 7th day. In accordance with the seasonal variations, a minimum of 28 to 30 days are required to complete the process of fermentation in *Kanjika* at present[9].

Table 1: Raw materials for *Kanjika*

S. No	Raw materials	Latin names	Part used	Quantity
1	Rice	*Oryza sativa*		100 g
2	*Saindhava lavana*	Sodium chloride	-	100 g
3	*Masha*	*Vigna mungo*	Seeds	25 g
4	*Sarshapa*	*Brassica nigra*	Seeds	50 g
5	*Haridra churna*	*Curcuma longa*	Tuber	25 g
6	Bamboo	*Bambusa vulgaris*	Leaves	25 g
7	*Jiraka churna*	*Cuminum cyminum*	Seeds	10 g
8	*Shunti churna*	*Zingiber officinale*	Tuber	10 g
9	*Shodhitha hingu*	*Ferula asafoetida*	Oleogum resin	5 g
10	*Sarshapa taila*	*Brassica nigra* (oil)	Seed Oil	22 ml
11	*Kulatha kwatha*	*Macrotyloma uniflorum*	Seeds	200 ml
12	Water	-	-	800 ml

No specific physicochemical parameters specific to *Kanjika* have been recommended by AYUSH or CCRAS till date. Since, it is an acidic fermentation product, pH, Brix, Specific gravity and Acid value were assessed and compared.

Comparative analysis of physicochemical parameters during different time intervals

Both the samples after completion of fermentation possessed the same organoleptic properties (Table 2). During fermentation, pH decreases with a simultaneous increase in acidity due to the accumulation of lactic and other organic acids as a result of microbial activity[1]. pH of KD_4 is significantly acidic when compared to KU_4. Increase in Brix value might be related to total soluble solids. Salt and other soluble materials that are formed during the process of fermentation might have contributed to the increased Brix value. Brix of the KD_4 is significantly high than KD_0. Specific gravity of the disturbed sample remained constant throughout the period of fermentation. Specific gravity remained constant in all the samples irrespective of keeping disturbed or undisturbed since the media used is water for all the samples invariably. Acid value showed significant decrease in KD_1 when compared to KD_4. (Table 2 & 3)

Comparative analysis of physicochemical parameters of disturbed and undisturbed sample after completion of fermentation

There has been significant increase in the Brix value in the undisturbed sample when compared to the K_0 and disturbed sample. A considerable increase in Brix value has been observed in the undisturbed sample when compared to the disturbed sample which might have occurred due to the varied rates of fermentation. pH has remained almost constant in KD_4 and KU_4. Specific gravity has shown slight increase in KU_4 when compared to KD_4. (Table 4) There has been no significant change in the acid value of the formulation irrespective of the way in which it is maintained throughout the process. (Table 2 & 3)

Table 2: Organoleptic characters of Undisturbed and Disturbed samples of *Kanjika*

Organoleptic Characters	KD_4	KU_4
Colour	Straw yellow	Slightly dark yellow
Odour	Acidic	Acidic
State	Liquid	Liquid
Taste	Sour	Sour

Table 3:Parameters of disturbed sample of *Kanjika*

Parameters	K_0	KD_1	KD_2	KD_3	KD_4
pH	6.21 at 27.9°c	5.05 at 27.2°c	3.38 at 27.8°c	3.26 at 28.7°c	3.24 at 29.3°c
Brix	7%	7.4%	8%	8%	8%
Specific gravity	1.04 at 27.9°c	1.042 at 27.2°c	1.042 at 27.8°c	1.042 at 28.7°c	1.042 at 29.3°c
Acid value	-	0.405	0.3635	0.312	0.3745

Table 4: Comparative analysis of Disturbed *Kanjika* sample and Undisturbed *Kanjika* sample

Parameters	KD_0	KD_4	KU_4
pH	6.21 at 27.9°c	3.24 at 29.3°c	3.31 at 29.3°c
Brix	7%	8%	11%
Specific gravity	1.04 at 27.9°c	1.042 at 29.3°c	1.056 at 29.3°c
Acid value	-	0.3745	0.279

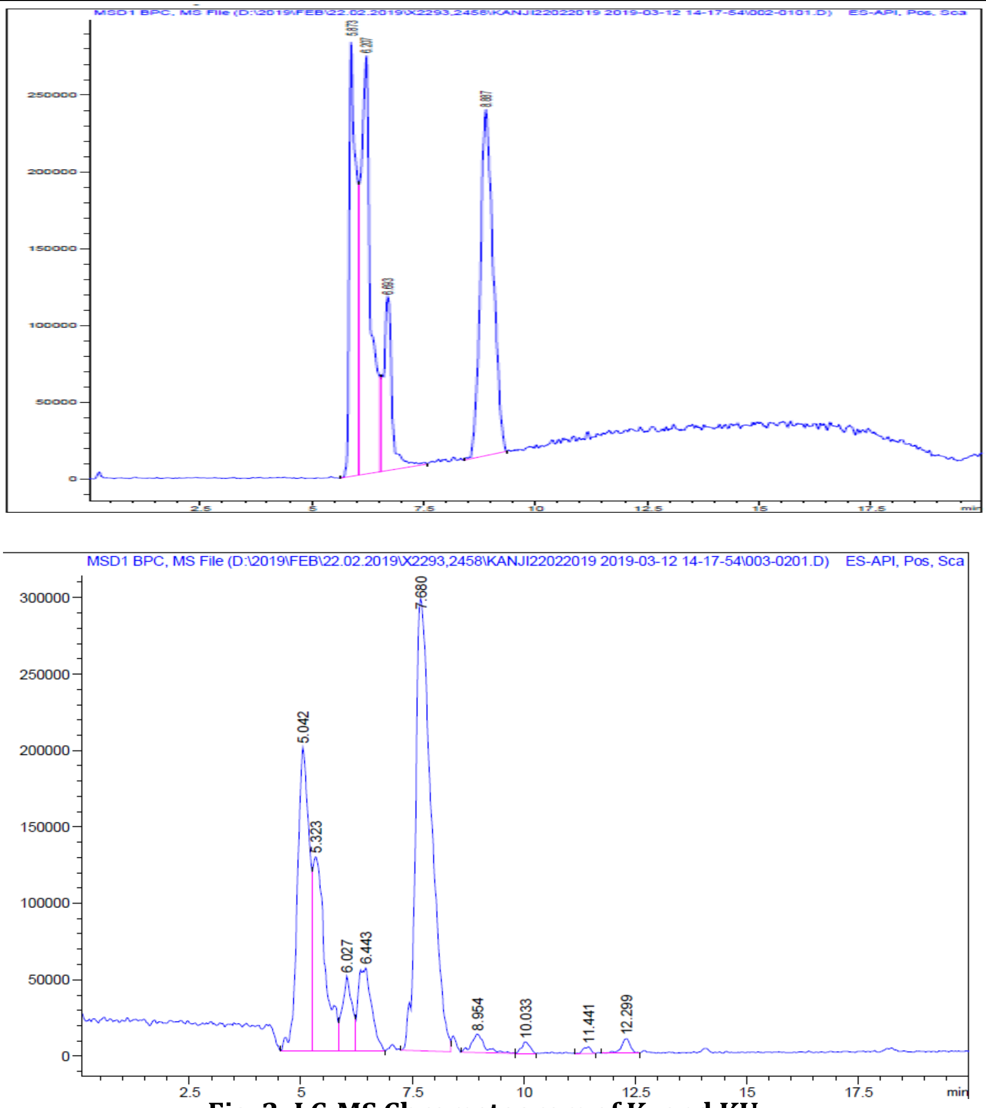


Fig. 2: LC-MS Chromatogram of K_0 and KU_4

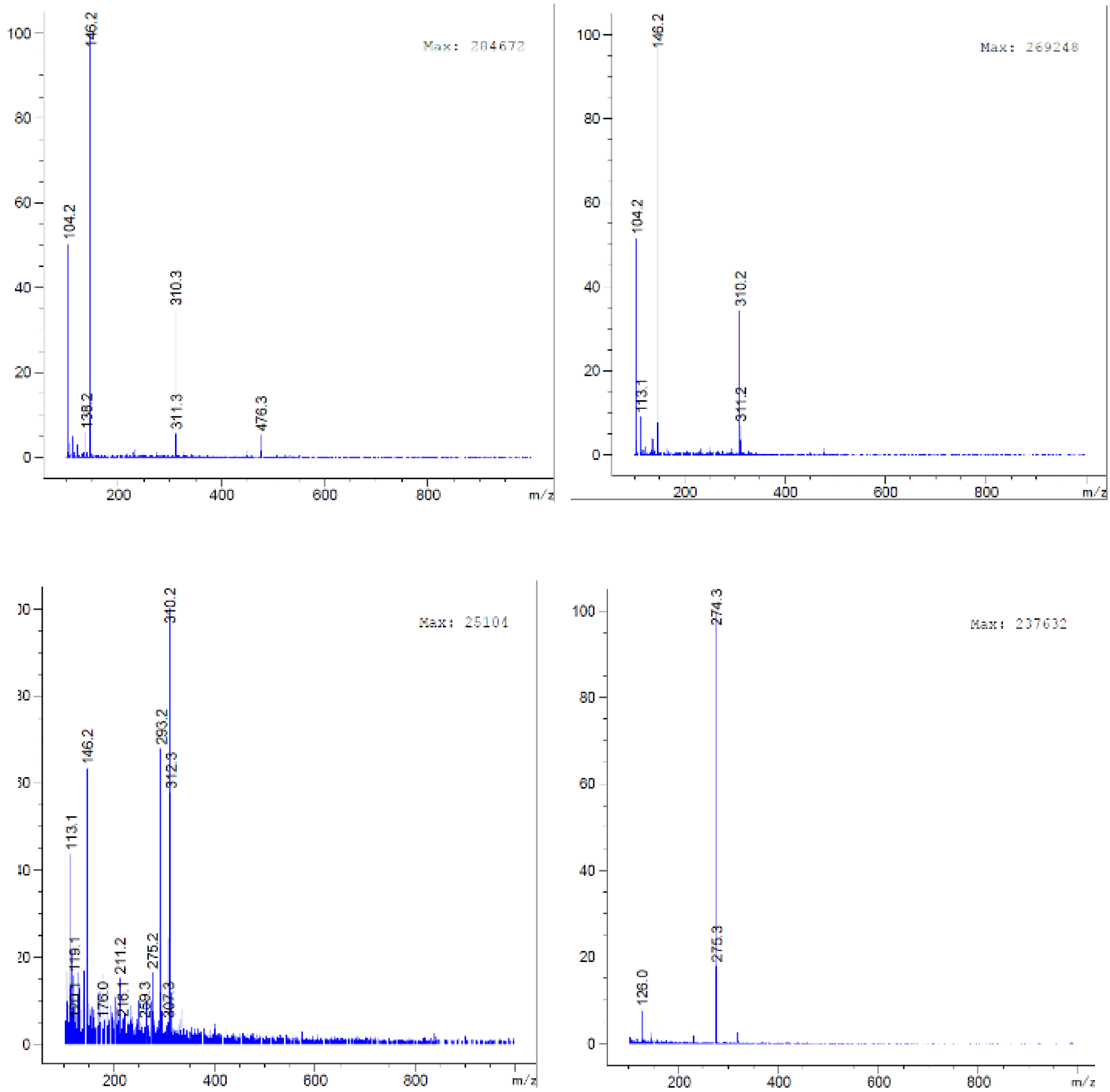
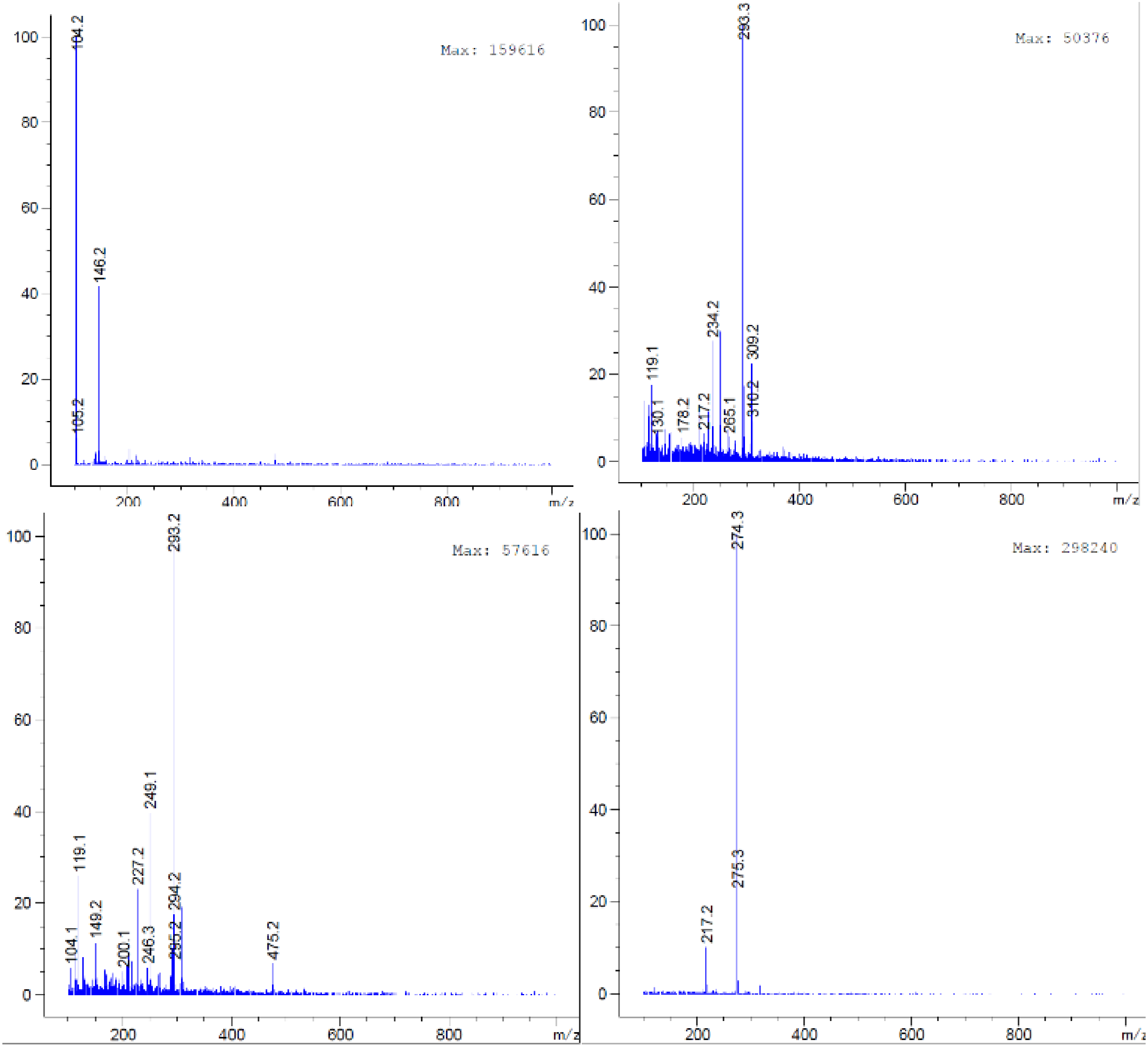


Fig. 3: Fragmentated MS spectra of K_0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146.2
105.2
Max: 50376
293.3
234.2
309.2
119.1
130.1
178.2
217.2
265.1
310.2
Max: 57616
293.2
249.1
119.1
227.2
294.2
149.2
104.1
200.1
246.3
295.2
475.2
Max: 298240
274.3
275.3
217.2
m/z

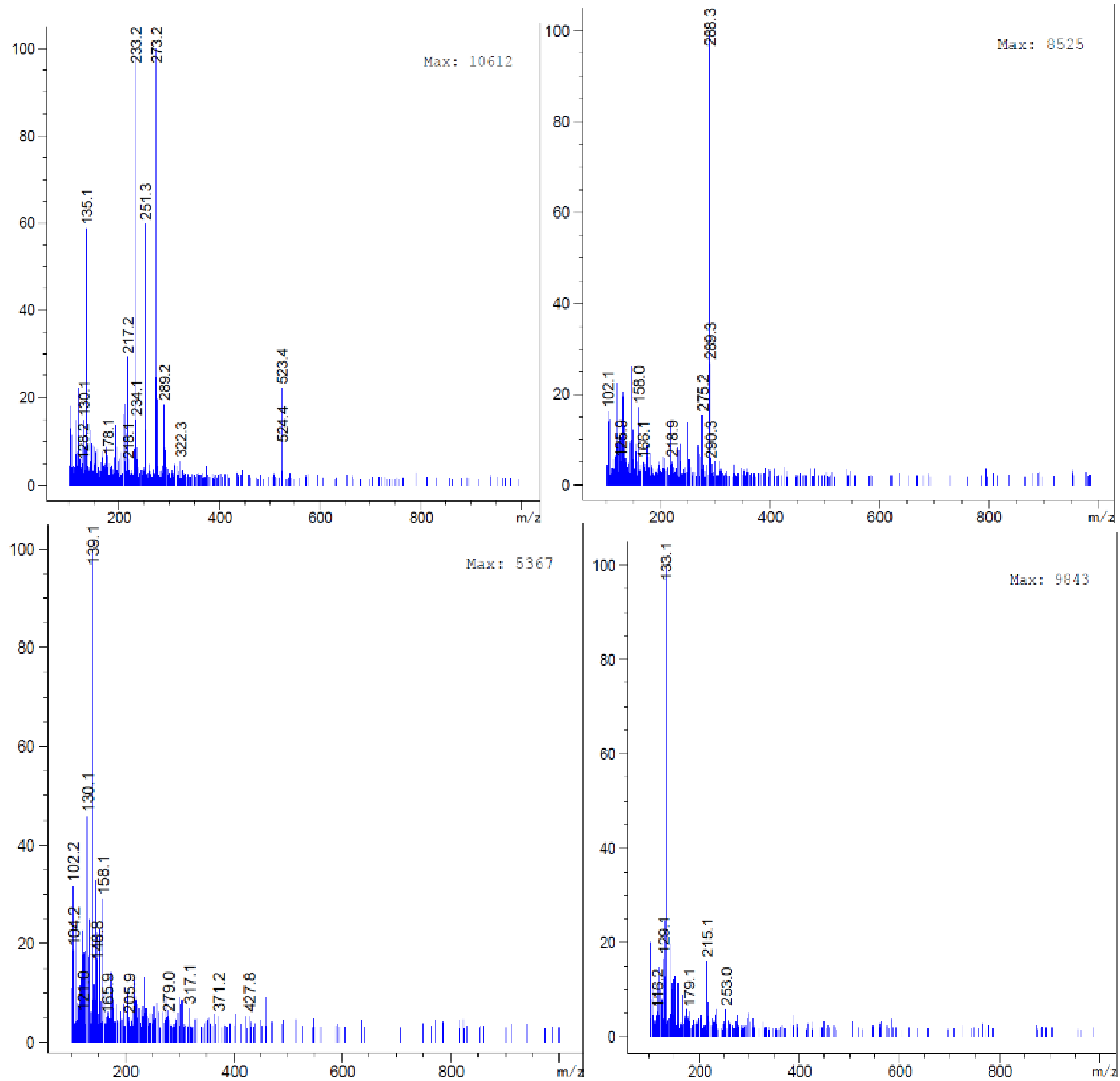

Fig. 4: Fragmentated MS spectra of KU_4

Comparative LC-MS analysis of preparation before initiation of fermentation and after completion of fermentation

LC-MS analysis of K_0 sample detected 4 peaks with retention times of 5.873,6.207,6.693 and 8.887 whereas KU_4 sample detected 9 peaks with retention times of 5.042, 5.323, 6.027, 6.443, 7.68, 8.954, 10.033, 11.441 and 12.299 respectively (Figure 2). Each peak of both the samples were then fragmented. Fragmenting spectrum of K_0 sample resulted in 3 fragmentation spectra with candidates mass (m/z) 146.2, 310.2 and 274.3. (Figure 3). 9 fragmentation spectra were obtained for KU_4 sample with candidates mass (m/z) 104.2, 113.1, 293.3, 293.2, 274.3, 273.2, 288.3, 139.1 and 133.1. (Figure 4). A spectrum database for organic compounds in SDBS application was used for the LC-MS spectrums interpretation. Spectrum interpretation indicated the presence of methyl glutaric acid with retention time of 5.873 and 6.207 of K_0 sample. Spectrum interpretation indicated the presence of lactic acid with retention time of 5.042 of KU_4 sample. These results were confirmed by each of the fragmentation pattern as figure 3 and figure 4.

Probable mode of interaction of the formulation with metals

The most probable mode of interaction of Kanjika with metals might be a contribution of various chemical processes initiated by the individual plant materials made use in preparing the formulation. Curcumin found in Curcuma longa, forms strong complexes with most of the known metal ions. Being a monobasic bidentate ligand, curcumin forms stable complexes with almost all metals and non-metals[10]. In spite of modifying the physico-chemical properties of curcumin, curcumin-metal complexes also affect the biological reactivity of the metals. It has been observed that complexation of metals with curcumin reduces the toxicity of metals[11–18]. In addition, LAB facilitates a complex process called metal ions quenching, which depends on two basic mechanisms, namely biosorption and bioaccumulation[19]. They may either possess the biosorption of many elements or specific elements depending on the species to which they belong[20]. Biosorption can be best described as the process of removal of metal ion, compounds and particulates from solution by biological material[21]. LC-MS analysis of the samples revealed the presence of several novel compounds in the final product when compared to the sample taken soon after preparation which were not identified. The presence of these peaks clearly indicates the importance of the fermentation process employed in the preparation of Kanjika. The presence of lactic acid in the final product was confirmed through LC-MS analysis. Researches on application of Lactobacilli in the biosynthesis of metal and metal oxide nanoparticles throw light on the utilisation of Kanjika in the pharmaceutical processing of Bhasma (incinerated metallic nanoparticles) [22]. This method of synthesis of nanoparticles using such probiotic species has also shown increased antioxidant capacity in experimental models [23]. Such research outcomes definitely provide a rationale on the usage of lactic acid fermented media. Such aspects can be probed into in the future works.

CONCLUSION

Pharmaceutical processing of drugs, especially those with a metallic or mineral nature has been practices for centuries now. The utility and pharmaceutical action of the media used remain as unknown territories which needs to be explored so that these methods can be well appreciated. Kanjika being one such media, employed in the processing of Bhasma was in the present study analysed to understand the probable biochemical processes taking place during its preparation and during it's interaction with metals and minerals. Further researches using higher analytical techniques must be carried out to understand the possible interactions and chemical reactions taking place in detail. Newer understandings on the same may open doors for applications of the same in the field of nanoscience and technology.

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