

Metabolic Profiling of Wastes from Raw and Ripe Mango Seeds to Comprehend Its Beneficial Medicinal and Cosmetic Properties for Commercialization

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Abstract

Mango (*Mangifera indica*) is a widely popular tropical fruit. When ripe, it is consumed either as fruit or juice, while raw mangoes are used in pickles, juice and curries. Apart from these, the seed kernels of ripe mangoes are in great demand by cosmetic and confectionary industries, while those from raw mangoes are used for medicinal purposes in traditional households of India. Although, the seed kernels have been used for various beneficial purposes, the inconsumable mango seed largely poses as a waste and biological pollutant. Therefore, it is pertinent to highlight the detailed composition and benefits of butter from various stages of mango kernel to highlight its commercial relevance. By using a combination of nuclear magnetic resonance spectroscopy and gas chromatography-mass spectroscopy based metabolic profiling, we studied the biochemical composition of different stages of mango kernel extract. We observed predominance of various types of fatty acids in all types of mango kernel with the ripe version showing the largest amount. The unripe versions though showed presences of a variety of aromats and antioxidants albeit at lower amounts. Our findings highlight that the unripe mango kernel extracts contains various aromats that have antimicrobial effects. India produces up to 23 million tonnes of mangoes every year and it has been estimated that it can produce up to 2.3 million tonnes of mango butter. Along with the fatty acids, these antimicrobials can be extracted as butter for commercial relevance thereby adding value to the seed kernel waste which otherwise pose as bio-pollutant.

Keywords: Mango seed kernel, Nuclear Magnetic Resonance spectroscopy, Gas chromatography, mass spectroscopy, metabolic profile

1. Introduction

Mango (*Mangifera indica*) is a tropical fruit that is widely grown in India and other warmer regions like South Africa, Pakistan, Bangladesh, Mexico, and few more countries. India accounts for more than 54% of the global production of mango estimated to be around 17-23 million tonnes Kg annually (FAO 2002; Ministry of Agriculture, Government of India (2013); Srikanth et al. 2015). The fruit comprises of a fleshy region and a stone, which consists of a hard shell and the kernel within. The fruit is a global delicacy widely consumed throughout the world. Refreshment drinks such as juices and milk-shakes are also very popular around the world. Due to its wide spread popularity, a lot of biochemical data (Gil et al., 2000; Khakimov et al., 2016) is available on the composition of kernel, especially its fatty acid (FA) content (Gunstone, 2007; Shobana and Rajalakshmi 2010). In comparison to its ripe counterpart, raw mango is widely used for culinary purposes, especially in India, where it is a major component of the pickling and chutney industries. Apart from this it is used as refreshment drink known as kairi panna in western part of the nation and the seed is used in South Indian curries called as thogayal and kozhambu. The seeds of mango are also used in traditional Indian medicine to treat gastric related problems. In fact, scientific reports have shown that extract of ripe mango kernels have antimicrobial activity (Alok et al., 2013; UNIDO) and can be used as food preservative (Pereira et al., 2011) as well. It is thus important to understand the composition of the various stages of kernel to realize its flavouring and pharmacological properties (Shobana and Rajalakshmi 2010; Leake, 1974).

Due to the overwhelming size of the industry (Ministry of Agriculture, Government of India (2013)) seeds from both raw and ripe mango pose a major environmental pollution problem. In many food industries, these wastes are dumped on sites nearby, where they accumulate and decay, producing foul odour. Similarly, biological degradation of mango stones is also a tedious process as it can take up to 8 months. It is thus important to identify and generate awareness of the different components present in mango seed kernels that can be used for various commercial purposes, thereby effectively minimizing pollution. The benefits of ripe mango kernel (RiMK) is known (though largely restricted to FA), and is used in cosmetic and food industries as a substitute to cocoa and shea butter (Kaphueakngam et al, 2009; Kittiphoom and Sutasinee, 2013).

Likewise, it is necessary to identify in details the biomolecules in raw mango kernel (RaMK) and the intermediary raw mango kernel (IrMK) that can benefit health, food, medicine and cosmetic industries. It has been estimated that India has a potential of extracting many million metric tonnes of Mango butter (FAO 2002).

In recent years, biophysical techniques such as Nuclear Magnetic Resonance (NMR) spectroscopy, Gas/Liquid - chromatography techniques and mass-spectroscopy (GC/LC-MS) techniques have greatly aided in rapid identification of various metabolites. In fact NMR and GC-MS/LC-MS are the most widely used tools in metabolomics analysis (Chen et al., 2014; Coen et al., 2008; Grosch et al., 2013; Jackels et al., 2014; Nobakht et al., 2016; Scalbert et al., 2009; Smolinska et al., 2012; Sumner et al., 2003; Ulrich et al., 2008; Zekeya et al., 2014). NMR spectroscopy offers a readily usable technique, which has sensitivity at nM concentrations. In comparison, chromatography-MS coupled techniques shows sensitivity at pM concentrations and contains a huge number of organic molecules in their database resulting in greater ability to identify compounds (Grosch et al., 2013; Sumner et al., 2003). Due to the pros of both the techniques, many research groups have resorted to applying them for rapid and reliable assessments (Chen et al., 2014; Grosch et al., 2013; Ly et al., 2005).

Using a combination of GC-MS and NMR spectroscopy, we have identified various components by metabolic profiling that are present in mango seed kernels of raw (RaMK), intermediate (IrMK) and ripe (RiMK) states. As mentioned above, the IrMK is the unripe version that is used by the pickling industry. The data on metabolites present in different mango seed kernels will provide us an understanding on the positive effects of each kernel type and serve as an important biochemical repertoire for further studies and commercial interests in medicine and cosmetic industries.

2. Materials and Methods

2.1 Sample Preparation

All three types of seeds; raw, intermediate and ripe were dried. The hard shell was removed and the kernel was used for the intermediate and the ripe seeds only, while the raw seed did not contain any hard shell and was used as it is. The kernel was finely chopped and 13.5 grams of each type was treated separately in a conical flask containing 7.5:10:20 ml of water:iso-butanol:chloroform for organic phase extraction (Nzikou et al, 2010). The mixture

was kept in dark for one week. The organic phase was separated from aqueous phase and was treated as described below prior to experimentation.

2.2 Nuclear Magnetic Resonance Spectroscopy

Five ml of the samples were dried in a hot air oven maintained at 75°C for 2 days. To characterize hydrophilic and lipophilic extracts of the sample, the dried preparation was suspended in 0.5ml D₂O (deuterium oxide, 99.9% D, Board of Radiation & Isotope Technology) and CDCl₃ (99.9%D Aldrich Chemicals Company), respectively. All the samples were measured at 25°C on an AVANCE III HD Ascend 500 MHz ((Bruker Biospin Switzerland) liquid state FT-NMR spectrometer coupled with a 5 mm BBFO probe, two rf-channels. All spectra were acquired at 298K (25°C). The ¹H-NMR spectra of Mango samples were recorded at constant temperature of 298K (25°C) using the Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence (Carr and Purcell, 1954). The NMR lock signal was obtained by adding D₂O and CDCl₃. The acquisition parameters of ¹H included 10.2 ms 300 pulse, 16 transients collected into 65 k data points with a spectral width of 10000Hz, an acquisition time of 3.28s and a relaxation delay of 1s. Prior to Fourier transformation, an exponential function corresponding to a line-broadening factor of 0.3 Hz was applied to the free induction decay (FID). The acquisition parameters of 1D ¹³C spectrum included 1.1s 90° pulse, 1024 transients collected into 65K data points with a spectral width of 29760 Hz, an acquisition time of 3.28s and a relaxation delay of 2s with ¹H decoupling using WALTZ-16 (Shaka et al., 1969). Prior to Fourier transformation, an exponential function corresponding to a line-broadening factor of 0.5 Hz was applied to the free induction decay (FID). ¹H-¹³C HSQC spectra were recorded with the settings: 48 transients, 1024x256 points (F2 x F1) and spectral width of 4348 Hz and 20830 Hz for F2 and F1, respectively. All data sets were linear predicted and zero filled once in the indirect dimension before Fourier transformation and final processing. ¹H and ¹³C NMR spectra were calibrated with respect to water and chloroform peaks at 4.8 and 7.11 ppm, respectively. All raw time domain FIDs were extracted to frequency domain spectrum using system supported TopSpin NMR software (version3.5, Bruker Biospin Ltd.).

2.3 Identification of Metabolites Based on NMR Spectroscopy

NMR spectra contain information about the effective hydrophilic and hydrophobic metabolites present in the sample. The NMR chemical shift peak lists were submitted to the database, Biological Magnetic Resonance Data Bank (Ulrich et al., 2008) (BMRB-<http://www.bmrwisc.edu/metabolomics/>) metabolomics webserver. Metabolites corresponding to the submitted resonances were matched and identified using chemical shift of metabolites at BMRB database. The program enlisted a range of organic compounds that may occur in the sample that were indexed with a score. Higher score reflected greater peak match between recorded spectra and chemical shifts in database.

2.4 Gas Chromatographic and Mass Spectrometric Analysis

Mango sample (1mg) was dissolved in 1mL of ethyl acetate and methanol (1:1v/v) at room temperature for 15 minutes followed by centrifugation (3000 g, 10 minutes) at 4°C. Samples

were analyzed with Shimadzu GC MS QP 2010 Ultra Gas chromatograph Mass Spectrometer. One μL sample was injected by auto sampler to Rtx 5 MS column capillary column coated with 5% phenyl cross-linked 95% dimethylpolysiloxane ($30\text{m} \times 0.25\text{mm}$ Id., $0.25\mu\text{m}$ film thickness). For GC/MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.99%) was used as the carrier gas.

Analyses were performed in the split mode with ratio 25 using helium gas carrier at 1.21 mL/min flow by direct injection method with injection volume of $2\mu\text{L}$ using ion source temp 230°C . Column temperature was maintained at 60°C for 10 minutes, then raised to 160°C at $10^\circ\text{C}/\text{min}$, held for 4 minutes then raised to 240°C at $8^\circ\text{C}/\text{min}$ and held for 6.0 minutes, and finally raised from 160 to 240°C , over the range for data collection was 10–900Da. Mass spectra were taken at 70 eV with 0.2s scan interval.

2.5 Metabolic Profiling Analysis Using GC-MS

The peaks that were separated from GC-MS were identified by Postrum Analysis software (Shimadzu) using the mass spectral databases of the instrument. Peak assignments were made using dual criteria of mass spectral database and linear retention index match. Retention indices of the eluted compounds were calculated on the basis of the standard alkane mixture (C11–C18). By comparing the retention indices and unique mass spectra with the reference library matches and literature retention indices, assignments were derived and considered for only those compounds with a matching factor of $>50\%$.

3. Results and Discussion

We analyzed the aqueous and organic phase of the extracts. Most of the compounds identified by both NMR (Figure 1 and 2) and GC-MS (Figure 3 and 4) showed a rich profile of fatty acids that accounts for more than 90% content in the RiMK (Kittiphoom, 2012). This is best appreciated from the NMR spectra in Figure 1, where one can observe intense chemical shift resonances for fatty acids in sample that was solubilized in CDCl_3 . The sample in D_2O (Figure 1A) showed similar peaks but with far lower intensities along with chemical shifts for polar groups in the 2-4.5 ppm range of $1\text{D } ^1\text{H}$ spectrum. In fact, when we compared the $1\text{D } ^{13}\text{C}$ spectra (Figure 1B and 1D), we noticed far greater signal intensities for the fatty acids and aromats in CDCl_3 over D_2O based sample, which is expected due to solubility of individual components. Furthermore, both the RiMK and IrMK samples shows a far diverse group of compounds that is readily visible in the $1\text{D } ^1\text{H}$ spectra in D_2O and CDCl_3 (details of which are provided below). Similarly, when we performed GC-MS based analysis, we observed that all three samples were rich in FAs (retention time of 20-23 min, see figure 3), but the compound list for RaMK was far smaller than the other two. While fatty acids accounted for the major fraction in all tested seed kernel types, the remaining myriad organic molecules that were present in low quantities constituted phenolics and flavonoids that impart bitter taste and have medicinal values; building blocks such as sugars, amino acids, nucleotides and vitamins; and silicaceous organics were found in seed coatings (Appendix, Tables 1 and 2).

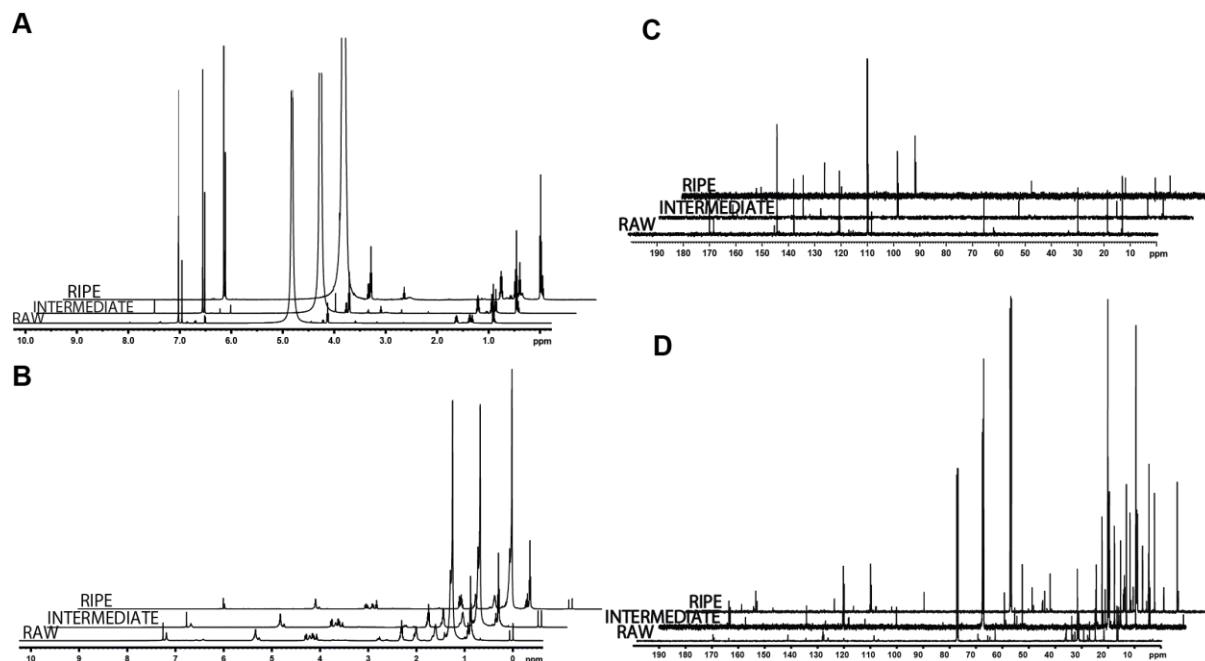


Figure 1. 1D ^1H (top) and ^{13}C spectra (bottom) for mango kernel samples in D_2O (left) and CDCl_3 (right).

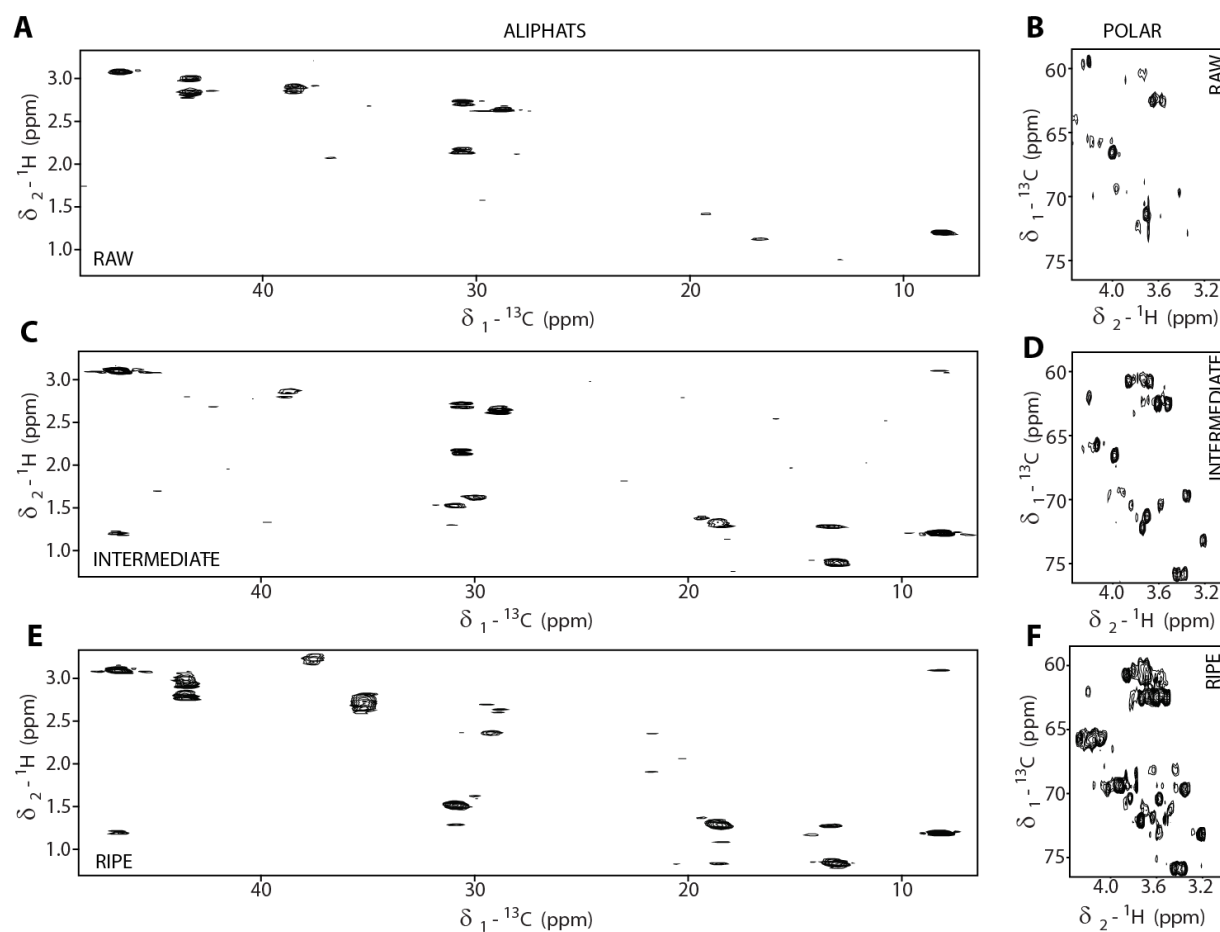


Figure 2. 2D ^1H - ^{13}C HSQC of the three stages of mango kernel in D_2O .

Comparison of metabolomics results between the two analytical techniques, we noticed few attributes; firstly, there were a handful of absolute compound matches between the two techniques, which was restricted to FAs such as stearic acid, palmitic acid and others. Secondly, albeit few absolute matches, the compounds identified by both techniques, NMR (appendix table1) and MS (appendix table2) belonged to similar functional groups. In all tested samples, based on GC-MS data (Figure 3 and 4), we did not notice the presence of molecules that are intrinsic for glucose, amino acid metabolism and TCA pathways. The NMR experiments on the other hand revealed the probable presence of these compounds but with low scores. This discrepancy may arise due to the number of molecules deposited in the database (Scalbert et al., 2009; Sumner et al., 2003). Perhaps, it may also reflect on the sampling and sensitivity of the technique itself, with GC-MS being a ~1000 fold more sensitive than NMR spectroscopy (Grosch et al., 2013; Sumner et al., 2003) resulting in matches with greater accuracy.

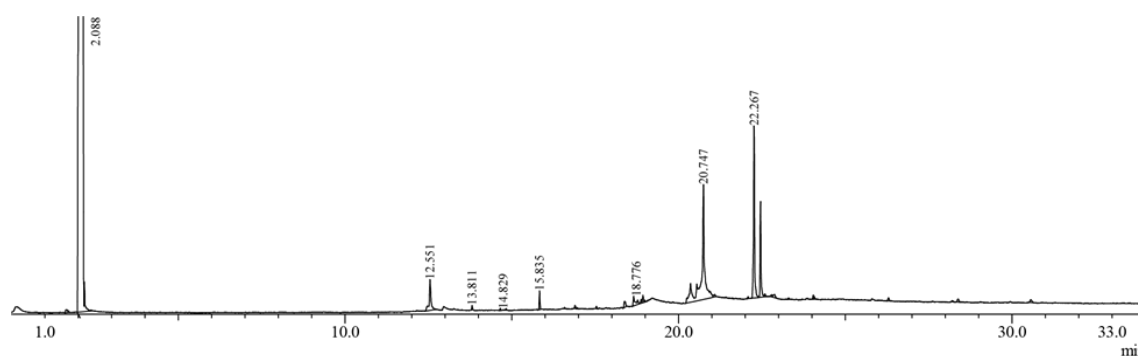


Figure 3. Gas chromatogram of analytes from the IM mango kernel sample. The individual fractions were subject to Mass spectroscopy (see Figure 4).

From the NMR experiments (appendix table1) we identified a variety of organic molecules in the IrMK and RiMK. The RaMK showed precursors for amino acid and lipid synthesis albeit with low scores. On the other hand the RiMK showed the presence of FAs such as decanoic acid, dodecanoic acid, glyceryl tridecanoate, myristic acid, palmitoleic acid and stearic acid; which is sought by the cosmetic industry and agrees well with previously reported data (Shobana and Rajalakshmi 2010). In addition to FAs, we noticed presence of building blocks like amino acids, sugars and nucleotides albeit with a lot lower score. In stark contrast, we observed amino acids, fatty acids and many of their precursors in the sample of IrMK with intermediate scores. These findings clearly illustrate that the RaMK contains few precursor metabolites, the IrMK showed a mixed presence of precursors and metabolites at the end of the cycle, while the RiMK is rich in various fatty acids.

Simultaneously, we analysed the GC-MS data to understand the contents of the kernel. As GC-MS is 1000-fold more sensitive than NMR we expected to see a diverse array of molecules. Indeed, we observed a wide variety of phytochemicals that have plenty of interesting medicinal properties (Table 1). In short, all three samples were rich in fatty acids and aromats, which is listed in table 2 and few interesting molecules have been described in section below. In the sample of RaMK we identified a couple of FAs and aromatic

compounds such as kojic acid (Bentley, 2006), which is commonly employed in cosmetics and mycotoxins; benzotriols (Kocacaliskan et al., 2006; Tom s-Barber n et al., 1990; Zekeya et al., 2014), barbital (Lopez-Munoz et al., 2005) and aromatic-fatty acids, which imparts bitterness and antimicrobial properties; hence it used in traditional Indian ayurvedic medicine, especially related to gastric problems. In the RiMK, we largely observed a variety of FAs and esters along with a few aromats. RiMK showed presence of FAs similar to the NMR experiments such as stearic acid, palmitic acid, oleic acid, etc; furthermore, it also contains a wide variety of esters that imparts fragrance to the extracted butter. These findings highlight the reason for mango butter being a highly sought product in food and cosmetic industry. In the IrMK, we observed a larger diversity of fatty and organic acids in comparison to the RiMK. The aromatic content of IrMK was also substantially higher as it contained antimicrobials derivatives from barbital (Lopez-Munoz et al., 2005), phtalic acid (Srinivasan et al., 2009), benzofuranone (Chiu et al., 2014; Ly et al., 2005) etc. IrMK encompasses properties of both RaMK and RiMK in terms of its antimicrobial properties (although the identified compounds were not the same) and biochemicals for cosmetic industries. In addition to these, we have enlisted (Table 1) some of the industrial applications of the various secondary metabolites in the above preparations.

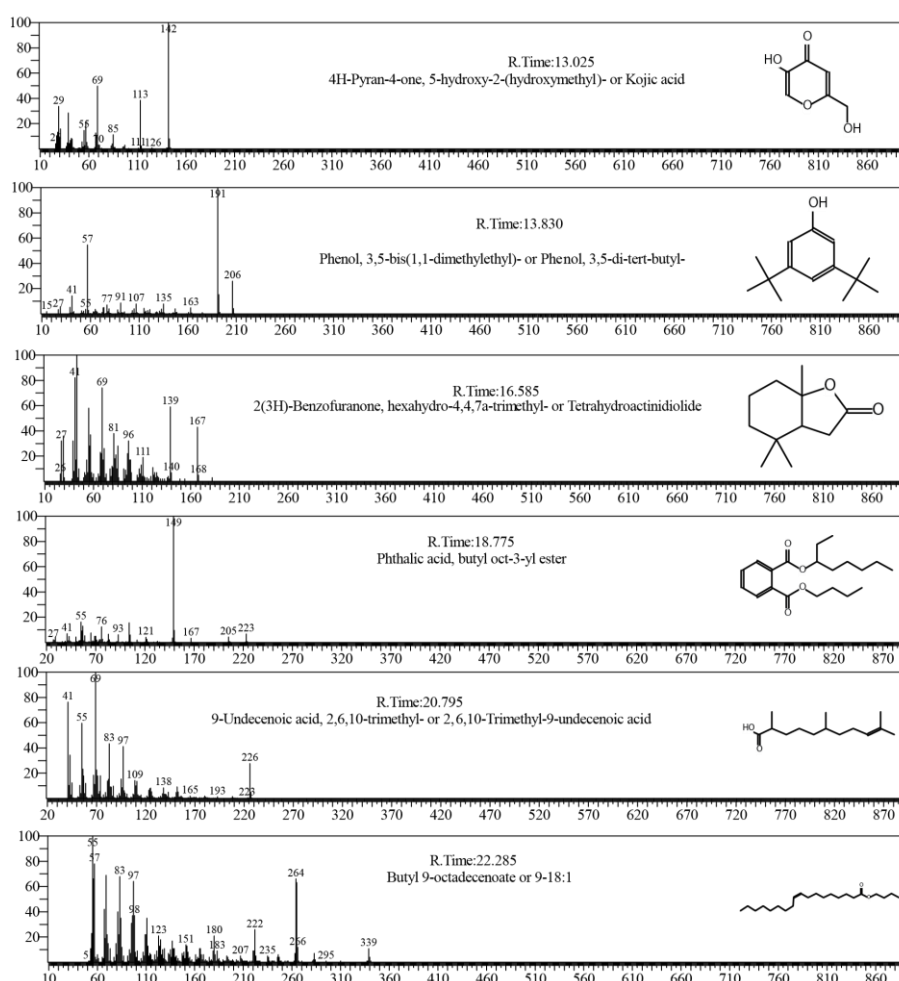


Figure 4. MS spectra of diverse group of compounds identified from the various fractions of gas chromatogram for IrMK (see Table 1 and appendix table2).

Table 1. Use of various secondary metabolites from different stages of mango kernel extract

Product	Applications
<p>Raw mango kernel:</p> <ol style="list-style-type: none"> 1. n-Eicosanoic acid; Stearic acid; Tetradecanoic acid anhydride; (3E)-3-Pentadecen-2-ol 2. (3Z)-3-Butyl-3-octen-2-one; 2,2-Dimethyl-5-(3-methyl-2-oxiranyl) cyclohexanone; 3-(Hydroxymethyl)-6-isopropyl-2-cyclohexen-1-one 3. 1,2,3-Benzenetriol; 1,2,4-Benzenetriol; Benzoic acid, 3,4,5-trihydroxy-, butyl ester; 3'-Hydroxyquinalbarbitone; 5-Methyl-1H-pyrazole-3-carboxylic acid 	<p>Emulsifiers for food, soap and cosmetic industry</p> <p>Odorants</p> <p>Medicine and pharmacology</p>
<p>Intermediate mango kernel:</p> <ol style="list-style-type: none"> 1. 2,3-Bis[(9E)-9-octadecenoyloxy]propyl (9E)-9-octadecenoate; Pentadecyl acrylate; Tetradecanoic acid, propyl ester; Heptadecafluorononanoic acid; octadecyl ester Nonadecyl 2,2,3,3,4,4,4-heptafluorobutanoate; 1-Propyldecyl methoxyacetate 2. Tetradecanoic acid, butyl ester; 3-Ethyl-5 Docosan-1-ol; 2,6,10-Trimethyldodecane 2,3-dihydroxypropyl ester; Pentadecyl acrylate; 14-Bromopentadecanoic acid; Octadec-9-enoic acid; Octadeca-1-ene; 1-Hydroxytridecane 3. (7E)-7-Methyl-7-heptadecene; 2-Oxo-octadecanoic acid; (9Z)-9-Tricosene; Phthalic acid, butyl oct-3-yl ester; Phthalic acid, butyl oct-3-yl ester; cyclobarbitol; 2,6,10-Trimethyl-9-undecenoic acid 4. 1-(+)-Ascorbic acid 2,6-dihexadecanoate ; 2,3-Dihydroflavone; 1-Methoxy-2-[1-(4-methoxyphenyl)-1-methylethyl]benzene; 5-Hydroxy-2-(hydroxymethyl)-4-pyrone; 4,4,7a-Trimethylhexahydro-1-benzofuran-2(3H)-one 	<p>Odorants and emulsifiers for food and cosmetic industry</p> <p>Emulsifiers, lubricants, surfactants for food, soap and cosmetic industry</p> <p>Medicine and pharmacology</p> <p>Antioxidants and odorants</p>
<p>Ripe mango kernel:</p> <ol style="list-style-type: none"> 1. Nonadec-1-ene; Stearic acid; cis-Oleic Acid; Palmitic acid; 2,3-Dihydroxypropyl (9E)-9-octadecenoate; 9-Octadecenoic acid; n-Pentadecanoic acid ;n-Eicosanoic acid; n-Octadecanol cis-9-Hexadecenoic acid; isobutyl hexadecanoate. 2. 1-(+)-Ascorbic acid 2,6-dihexadecanoate; 2,6-Bis(tert-butyl)phenol; 2,4-Di-tert-butylphenol; 2,2-Bis(3-methyl-4-hydroxyphenyl)propane; 2-Hydroxycyclopentadecanone. 	<p>Emulsifiers for food, soap and cosmetic industry</p> <p>Odorants</p> <p>Antioxidants and odorants</p>

4. Conclusion

India is the largest producer of mangoes globally accounting for more than 50% of the production amounting up to 23 million tonnes Kg per annum (Ministry of Agriculture, Government of India (2013); Srikanth et al. 2015). As the mango kernel accounts for nearly 40-65% of the weight and an extraction efficiency of 10%, indicates that at least 2.3 million tonnes Kg of mango butter can be readily extracted. This work highlights that the kernels obtained from intermediate stage mango is rich in biochemical in comparison to the ripe and raw versions. Herein, we show that the reason raw and intermediate mangoes are used for culinary purposes and ayurvedic medicine applications is due to the presence of aromatic compounds belonging to phenolics (Akagawa and Suyama, 2001; Zekeya et al., 2014) and flavonoids (Zekeya et al., 2014) that show antimicrobial properties and may also add to the flavour. On the other hand, we have shown that the RiMK contains very few aromats but a variety of FAs akin similar reports by other groups, which is required by the cosmetic industry. But IrMK also contains these different classes of FAs albeit ~3 fold lower than its ripe counterpart by comparing the gas chromatograms.

Although, the overall fatty acid content in the IrMK butter is lower than RiMK, it is rich in anti-oxidative and antimicrobials. Similar to RiMK butter extraction, IrMK butter can be readily extracted by using press filtration, organic solvent extraction (Gunstone, 2007; Nzikou et al, 2010; Kittiphoom and Sutasinee 2013) or distilling with supercritical CO₂ (Akanda et al. 2015). Like RiMK butter, IrMK butter will also find value in confectionaries (Ashoush and Gadallah, 2011; Pereira et al., 2011) and cosmetic industry (Kaphueakngam et al, 2009) due to similar content. The dried stone that is left behind after the entire process can be used either as mulch, cattle feed (FAO 2013; UNIDO) or for cultivation of mushrooms (Stamets, 1993) thereby reducing environmental contamination and at the same time adding value to the product.

Interestingly, the intermediate stage kernel is used by the pickle industry, where the seed is obtained as a by-product and is usually discarded. An average medium-scale pickle factory in India can generate up to 500 Kg of mango stone/day for a period of three months (March-May). If one were to assume loss in weight due to stone and extraction process yielding only 10% butter, it still offers a lucrative 50 Kg of mango butter daily, which sells at ~400 INR/Kg (5 Euros/Kg). This results in a total of 4.5 tonnes Kg of mango butter (1.8 million INR (200,000 Euros)) during the three months pickling season. According to the reports by UN and FAO, the potential of commercializing mango butter is huge and greatly untapped. It is thus pertinent that one highlights the biochemical composition of IrMK to the industry on the valued product thereby also reducing biological waste.

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