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Metabolite profiling of mango cv. Kesar leaf using gas chromatography-mass spectrometry

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Abstract
Mango Cv. Kesar is most popular variety of Gujarat. The leaf metabolite profiling of this cultivar carried out using highly used platform Gas chromatography-Mass spectrometry (GC-MS). The leaf sample metabolites were extracted and derivatized for metabolite profiling and identified on the bases of Retention time (min) and mass to charge ratio (m/z). Theses metabolites were classified in organic Acid (41% of total metabolites), Sugars (32%), other class (18%) and smallest class sugar alcohol (9%). Metabolite profiling of Mango Cv. Kesar leaf revealed that, the potential metabolites have their significance function in plant metabolism, plant protection against biotic and abiotic stress and also important for human health.

Keywords: GC-MS, mango Cv. Kesar, metabolites, organic acids, sugars, sugar alcohols

Introduction
The metabolic networks in higher plants are highly complex and multiplex biochemical steps. Metabolomics approaches used to assess the natural variation in metabolite content in individual plants, with great potential for the improvement of the compositional quality of crops. Metabolomics analysis is typically performed by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), including gas chromatography GC-MS, liquid chromatography LC-MS, capillary electrophoresis CE-MS among these platforms GC-MS is one of the mostly widely used analytical methods for metabolomics. Metabolite profiling is a fast growing technology and is useful for phenotyping and diagnostic analyses of plants. It is also rapidly becoming a key tool in functional annotation of genes and in the comprehensive understanding of the cellular response to biological conditions. (Gullberg *et al*., 2004; Schauer and Fernie, 2006) [8, 18]. Mango is the national fruit of India, known as the 'King of Fruits'. It is one of the most important and popular Asian fruits. Mango is popular due to its excellent flavour, delicious taste, delicate fragrance, attractive colour and nutritive value which make at rank among the best fruits of world. It is believed that mangoes originated in Northeast India. Mango (*Mangifera indica* L.) universally considered to be one of the finest fruits, and is an important crop in tropical and subtropical areas of the world. Major mango growing countries are India, China, Thailand, Pakistan, Australia, Indonesia, Bangladesh, Philippines, Nigeria, Myanmar and Egypt. There are about 1500 varieties of mango in the world from which about 1200 are found in India (Krishnan *et al*., 2009) [10]. The most popular cultivar grown around Gujarat state is Mango cv. Kesar. Kesar is characterized by its golden color with green over tones, and its unique flavour. The fruit is slightly smaller compared to the Alphanso variety. The fruits are medium to large sized (250-325 g per fruit), oblong in shape with an attractive light apricot-yellow color. The taste is very good and sugar/ acid blend is excellent. The cultivar is free from spongy tissue disorder and malformation. Tree bear excellent quality fruits with saffron coloured pulp when ripe and delicious. Excellent for table purpose fruits, medium sized with fiber-less stone. The “Kesar” fruit has 18 to 22 percent T.S.S., 0.25 to o.29 percent acidity and 10.5 to 12.0 percent total sugars with storability of 15 to 20 days (Singh, 1960; Chovatia *et al.*, 1995) [20, 6]. Ample information on Mango cv. Kesar leaf metabolite profiling available. Here, we characterized the contribution of metabolite profiling of Mango Cv. Kesar leaf and this information could be valuable to characterize certain aspects of genetic traits, which in turn will be useful for breeding programs and diagnostic/treatments aspects in future.

Materials and Methods

Mango cv. Kesar leaf sample was procured from Fruit research station, Sakkarbaug Farm, Junagadh Agricultural University, Junagadh, Gujarat, India. The experiment was carried out at department of Biotechnology, Junagadh agricultural University, Junagadh, Gujarat.

Extraction and derivatization of metabolites

Metabolite profiling study was performed using GC–MS. Metabolites were extracted as described by Lisec *et al*. (2006) [12] with minor modifications.

1. Leaf tissues (150 mg) were homogenized with pre-chilledmortar-pestle in 3 ml of 100% HPLC grade methanol (precooledat − 20 °C).
2. The mixture was shaken for 10 min at70 °C in a water-bath at 950 rpm and centrifuged for 10 minat 11,000 *g*. The supernatant was transferred to a Schott GL14 glass vial and 1.5 ml of chloroform (− 20 °C) wasadded.
3. After that 3.0 ml of dH2O (4°C) was added and vortexedfor 10s.
4. Again, the mixture was centrifuged for 15 minat 2200 *g* and the upper phase (polar) and lower phase (nonpolar) phase were transferred into a separate test tube.
5. Bothpolar and nonpolar phase were dried in a nitrogen stream. Extracted metabolites were derivatized as described by Sanimah *et al.* (2013) [17] with minor modifications.
6. The dried extracts were re-dissolved in 50 µl of pyridine and sonicated for 10 min.
7. Then, 100 µl of methoxyamine HCL (20 mg ml−1in pyridine) was added and vortexed for 30s.
8. The mixtures were then sonicated again for 5 min and incubated with constant agitation for 90 min at 37°C.
9. The trimethylsilylation (TMS) step was performed by adding 250 µl *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) to the extracts and vortexed for 30s.
10. Mixtures were incubated for 1 h at 37 °C for derivatization.

GC‑MS analysis

For GC–MS analysis, 1 µl of derivatized extract was injected into a DB-17MS capillary (30 m × 0.25 mm). The inlet temperature was set at 280 °C. After a solvent delay for 5 min, initial GC oven temperature was set at 100 °C; after injection for 1 min, the GC oven temperature was raised to 290 °C.

The injection temperature was set to 280 °C and ion source temperature was 230 °C. Helium was used as the carrier gas with a constant flow rate set at 1 ml/min. The measurement was performed with electron impact ionization (70 eV) in the full scan mode (m/z from 50 to 700). Metabolites were putatively identified by matching their mass spectra to spectra in NIST 14 library (National Institute of Standards and Technology, Gaithersburg, MD, USA). Pre-processing of total ion chromatograms (TIC) such as baseline correction, alignment, peak picking, and integration were performedusing the ACD/Spec Manager v.12.00 (Advanced Chemistry Development, Inc., ACD/Labs, Toronto, Canada). CSV comma delimited files were created for data analysis.

Results and Discussions

Total 85 different metabolites were identified in leaf of Mango Cv. Kesar by GS-MS**.** These metabolites were identified on the bases of their Retention Time (min) and m/z (mass to charge) ratio The GCMS chromatogram obtained from leaf of Mango Cv. Kesar is presented in Fig. 1.

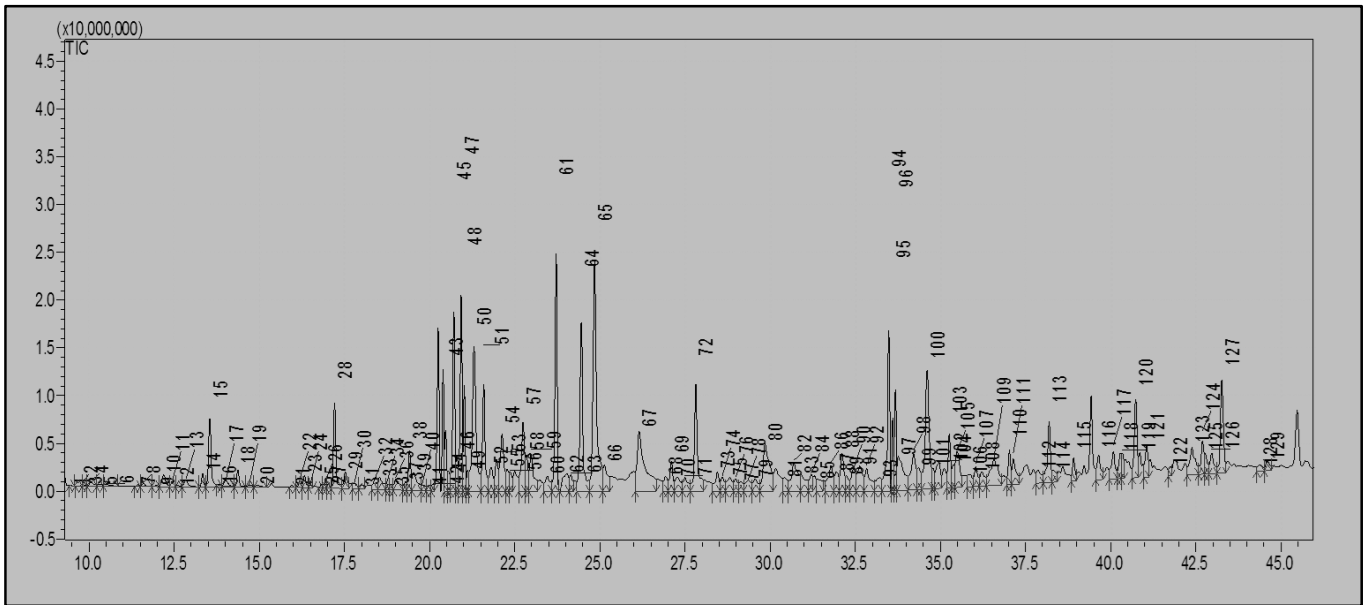

Fig 1: GCMS chromatogram of Mango Cv. Kesar leaf.

Functional classification and significance

The largest class was organic acid followed by, sugar, other classes and sugar alcohol (Fig. 2, Table 1). The largest class "Organic acid" comprised 41%. Organic acid involved at the cellular level for several biochemical pathways at the whole plant level in modulating adaptation to the environment (Lopez-Bucio *et al.,* 2000) [13]. This class includes few of the well known metabolites putatively known for their medicinal as well as economic values further strengthening the medicinal and economic properties of Kesar leaf. Among these organic acids, 1-Cyclohexene-1-carboxylic acid (RT 21.018) also known as Shikimic acid, a natural organic compound is generally utilized as a precursor for industrial synthesis of the antiviral Oseltamivir drug, chemotherapy of cancerous diseases, used as herbicides and antibacterial agents and for benzene-free production of phenol (Bochkov *et al.*,

2012). Threonic acid (2, 3, 4-Trihydroxybutyric acid, RT 14.367) is a sugar acid derived from threose, L-isomer is a metabolite of ascorbic acid (Englard and Seifter., 1986) [7]. One study suggested that because L-threonate inhibits DKK1 expression *in vitro*, it may have potential in treatment of androgenic alopecia (*Kwack et al.*, 2010) [11]. Other important members of this group were 2-Butenedioic acid, 2-Pentenedioic acid, Benzoic acid, ribonic acids, malic acid, L-Ascorbic acid etc. have their particular role in metabolism, growth and development of plant.

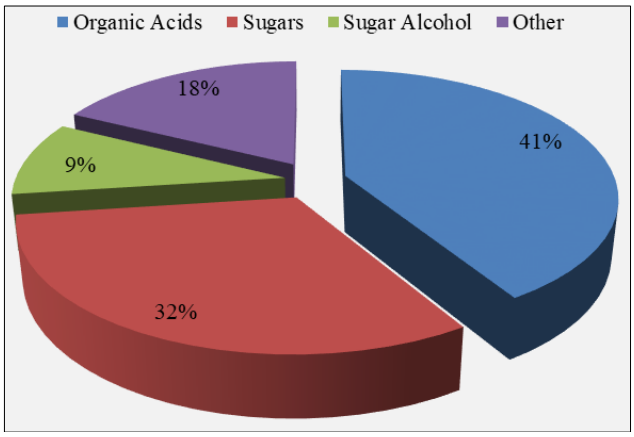

Fig 2: Functional classification of metabolites. All the metabolites identified by GC-MS analysis was classified into four groups as represented in the pie chart

Second largest class of metabolites was the "Sugars", which comprised 32% of total metabolits. Sugars role as providers of carbon and energy and fulfills signaling role in coordination with hormonal signaling pathways (Rolland *et al*., 2006) [16] controlling various plant physiological processes, including innate immunity (Bolouri Moghaddam and Van den Ende, 2012) [4]. Glucose, fructose, Galactose, maltose, ribose, turanose, melibiose andtheir different variants were present in leaf of Mngo Cv. Kesar. These are natural sugar derivatives and their different isoforms or variants were present at different retention time which given in Table 1.

The third largest class of metabolites was the "Other classes" which covers 18% of total metabolites, which includes various intermediate products of different metabolic pathways and their byproducts. Few of the metabolites and/or their isoforms eluted at different retention time in given in Table 1. This class includes 1-Monooleoylglycerol trimethylsilyl ether, Furanone, nucleoside uridin and psudouridin and alcohol derivatives. Uridine, a nucleoside was omnipresent across the wild type and transgenic lines. It contains a uracil attached to a ribose ring (known as a ribofuranose) via a β- N1-glycosidic bond. Uridine plays a major role in linking the glycolysis pathway with that of galactose and the conversion of galactose to glucose. Uridin containing drug Souvenaid® has demonstrated cognitive benefits in patients with mild Alzheimer's disease (Ritchie *et al.*, 2014) [15]. Monobe *et al.* (2003) studies suggested metabolite Pseudo uridine may offer protection from radiation. Furanones are also important as naturally occurring flavor compounds. They are found in many fruits including strawberry, pineapple, raspberry, grapes, tomato, kiwi and mango and many contribute caramel-like flavor notes of these fruits (Schwab and Roscher 1997) [19]. Ripe mango fruits contain high amounts of furaneol (4-hydroxy-2, 5-dimethyl-3(2H)-furanone) and its methyl ether, mesifuran (2, 5-dimethyl-4-methoxy-3(2H)-furanone). The fruits of cultivar Alphonso contained higher amounts of these two compounds than those found in any other cultivar (Pandit *et al*. 2009) [14]. Squalene (2, 6, 10, 14, 18, 22-Tetracosahexaene, 2, 6, 10, 15, 19, 23-hexamethyl-, (all-E)-, RT 40.733) has some beneficial properties when consumed either from food or as a supplement. These include potential anticancer effects, protective and moisturizing role in the skin, antioxidant activity and it may even improve the immune response to vaccines.

Furthermore, the smallest class of Mango Cv. Kesar leaf metabolome encompasses sugar alcohols. Sugar alcohols are reduced forms of aldose or ketose monosaccharide (Brimacombe and Webber, 1972) [5]. Mannitol, glucitol, myo-inositol and arabitol were the sugar alcohols found to be present in the leaf metabolome. Sugar alcohols are a type of carbohydrates called "polyols" and are used as subdued calorie sweetener, and often in combination with high intensity artificial sweeteners to counter the low sweetness. These sugar substitutes offer fairly low calories than table sugar (sucrose), primarily due to its low absorption and may even have a small laxative effect. Besides this function sugar alcohol also involved in osmoprotective mechanism of plant against Abiotic stress such as cold, drought, salt stress. The transgenic tobacco containing mannitol 1-phosphate dehydrogenase (mtlD) from *Escherichia coli* resulted in mannitol production and a salinity-tolerant phenotype (Tarczynski *et al.*, 1993; Thomas *et al*., 1995) [21, 22]. The cyclitols such as myo-inositol have shown better stress protection. The myo-inositol is synthesized by myo-inositol 1-phosphate synthase and is induced by salinity (Ishitani *et al.*, 1996) [9]. The transgenic *Arabidopsis* plants overexpressing *G.max* IMT have displayed improved tolerance to dehydration stress treatment and high salinity stress treatment (Ahn *et al.*, 2011) [1]. The transgenic *A. thaliana* plants expressing halophyte *Mesembryanthemum crystallinum*myo-inositol-O-methyltransferase (Imt1) in response to cold stress have shown elevated cold tolerance. In addition, accumulation of sugar alcohols like mannitol or sorbitol has been linked to stress tolerance (Arbona *et al*. 2013) [2].

Table 1: List of Metabolites in leaf of Mango Cv. Kesar

Organic Acids		
Name	**RT**	**m/z ratio**
1-Cyclohexene-1-carboxylic acid, 3, 4, 5-tris [(Trimethylsilyl)oxy]-, trimethylsilyl ester, [3R-(3.alpha., 4.alpha., 5.beta.)]-	21.018	204.15
2, 3, 4-Trihydroxybutyric acid tetrakis(trimethylsilyl) deriv.	14.367	73.1
2-Butenedioic acid (E)-, bis(trimethylsilyl) ester	9.749	245.15
2-Butenedioic acid (Z)-, bis(trimethylsilyl) ester	10.499	147.15
2-Pentenedioic acid, 2-[(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	22.98	73.1
2-Trimethylsilyloxyheptanoic acid, trimethylsilyl ester	9.472	173.25
3-Ethyl-3-hydroxyglutaric acid triTMS	22.743	73.1

3-Hydroxy-3-(4'-hydroxy-3'-methoxyphenyl)propionic acid, tri-TMS	37.868	297.15
Benzoic acid, 3,4,5-tris(trimethylsiloxy)-, trimethylsilyl ester	24.844	73.05
Benzoic acid, 3,4,5-tris[(trimethylsilyl)oxy]-, propyl ester	29.352	73.05
Benzoic acid, 3-methyl-2-trimethylsilyloxy-, trimethylsilyl ester	24.455	281.15
Benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester	18.41	73.1
Butanedioic acid, bis(trimethylsilyl) ester	9.877	147.15
D-Gluconic acid, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, trimethylsilyl ester	19.009	73.1
D-Glucuronic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	31.212	73.05
Gluconic acid, gamma.-lactone, 2-methoximine, tri(trimethylsilyl)-	24.03	73.1
Gluconic acid, gamma.-lactone, 5-methoximine, tri(trimethylsilyl)-	25.13	73.1
Gluconic acid, 2-methoxime, tetra(trimethylsilyl)-, trimethylsilyl ester	17.784	73.1
Gluconic acid, 2-methoximine, tetra(trimethylsilyl)-, trimethylsilyl ester	28.816	73.1
Gulonic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl)-, lactone	19.782	73.1
Hexacosanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	40.08	439.5
Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	35.384	73.05
Hexadecanoic acid, trimethylsilyl ester	26.151	73.05
Idonic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl)-, lactone	22.36	73.1
L-Ascorbic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl)-	24.14	73.1
Malic acid, tris(trimethylsilyl) ester	13.543	73.1
Malonic acid, bis(2-trimethylsilylethyl ester	21.115	73.1
Mannonic acid, 2,3,4,6-tetrakis-O-(trimethylsilyl)-, lactone	22.841	73.1
Nonanoic acid, trimethylsilyl ester	10.061	73.1
Octanedioic acid, bis(trimethylsilyl) ester	19.989	73.1
Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	18	73.1
Propanoic acid, 2-methyl-2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	11.989	73.1
Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	18.528	73.1
Talonic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl)-, lactone	38.911	73.1
trans-9-Octadecenoic acid, trimethylsilyl ester	29.85	73.05
Sugar		
.alpha.-D-Mannopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	32.832	204.1
.beta.-D-Galactofuranose, 1,2,3,5,6-pentakis-O-(trimethylsilyl)-	22.277	217.15
.beta.-D-Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	31.783	204.1
2-Deoxy ribose per-TMS II	15.956	73.1
4-Ketoglucose, bis(O-methyloxime), tetrakis(trimethylsilyl)	12.446	73.1
4-Ketoglucose, methoxy, silyl	21.588	73.1
Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-	19.534	217.15
Arabino-Hexos-2-ulose, 3,4,5,6-tetrakis-O-(trimethylsilyl)-, bis(dimethyl acetal)	12.581	73.1
d-Arabinose, tetrakis(trimethylsilyl)-	19.295	73.1
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	20.709	73.1
D-Fructose, 6-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.alpha.-D-glucopyranosyl]-1,3,4,5-tetrakis-O-(trimethylsilyl)-	44.376	73.1
D-Galactose, 6-deoxy-2,3,4,5-tetrakis-O-(trimethylsilyl)-, O-methyloxime	17.011	117.15
D-Mannopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	27.498	73.05
D-Turanose, heptakis(trimethylsilyl)-	34.608	73.05
D-Xylopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)-	28.452	73.1
Glucose oxime hexakis(trimethylsilyl)	20.544	73.1
Inosose, 2-desoxy-, O-methyloxime, tetrakis-O-(trimethylsilyl)-	21.802	73.1
Maltose, octakis(trimethylsilyl)-	33.223	204.1
Melibiose, octakis(trimethylsilyl)-	29.563	204.15
.alpha.-D-Galactopyranoside, methyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	30.694	204.1
.alpha.-D-Galactoside, methyl tetrakis-O-(trimethylsilyl)-	22.127	73.1
.alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	33.483	361.2
.alpha.-D-Glucopyranoside, methyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	30.423	204.1
.alpha.-D-Mannopyranoside, methyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	41.75	204.15
2-O-Glycerol-.alpha.-d-galactopyranoside, hexa-TMS	28.989	204.1
Glycoside, alpha.-methyl-trtrakis-O-(trimethylsilyl)-	20.798	73.1
Hydroquinone-.beta.-d-glucopyranoside,pentakis(trimethylsilyl)-	35.26	73.05
Sugar alcohols		
Arabinitol, pentakis-O-(trimethylsilyl)-	16.326	73.1
D-Glucitol, 6-deoxy-1,2,3,4,5-pentakis-O-(trimethylsilyl)-	16.827	73.1
D-Mannitol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	20.926	73.1
D-Myo-Inositol, 1,2,4,5,6-pentakis-O-(trimethylsilyl)-, bis(trimethylsilyl) phosphate	30.911	73.05
Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-, scyllo-	23.714	73.1
Myo-inositol, 5-deoxy-1,2,3,4,6-pentakis-O-(trimethylsilyl)-	18.877	73.1
Per-O-trimethylsilyl-(3-O-.beta.-d-mannopyranosyl-d-glucitol)	42.387	73.1
Ribitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	21.302	73.1
Other classes		
(4-Hydroxy-3-methoxyphenyl)ethylene glycol tris(trimethylsilyl) ether	39.646	297.2
1-Monooleoylglycerol trimethylsilyl ether	38.308	129.1

2(3H)-Furanone, dihydro-3,4-bis[(trimethylsilyl)oxy]-, cis-	14.931	73.1
2(3H)-Furanone, dihydro-3,4-bis[(trimethylsilyl)oxy]-, trans-	12.192	73.1
2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	40.733	69.1
Butanal, 2,3,4-tris[(trimethylsilyl)oxy]-, O-methyloxime, [R-(R@,R@)]-	23.454	73.1
Butanal, 2,3,4-tris[(trimethylsilyl)oxy]-3-[[(trimethylsilyl)oxy]methyl]-, O-methyloxime, (S)-	16.492	73.1
Butane, 1,2,3,4-tetrakis[(trimethylsilyl)oxy]-	11.437	73.1
Octacosanol trimethylsilyl ether	42.772	467.55
Pseudo uridine penta-TMS	14.013	73.1
Salbutamol, N-trifluoroacetyl-o,o,o-tris(trimethylsilyl)deriv.	42.965	369.25
Silane, [(3,7,11,15-tetramethyl-2-hexadecenyl)oxy]trimethyl-	27.816	143.15
Silane, [[2-[3,4-bis[(trimethylsilyl)oxy]phenyl]-3,4-dihydro-2H-1-benzopyran-3,5,7-triyl]tris(oxy)] tris[trimethyl-, (2R-cis)-	41.053	73.1
Trimethylsilyl ether of glycerol	27.277	73.05
Uridine, 2',3',5'-tris-O-(trimethylsilyl)-	19.408	73.1

Conclusion

GC-MS based metabolite profiling approach was used to evaluate the metabolic content of Mango Cv. Kesar. The results from our present study demonstrated the presence of 85 metabolites including organic acids, Sugars, Sugar alcohols and others. The identified potential metabolites presence in leaf extract may be responsible to exhibit different biological significance in the agriculture, food industry, pharma industry etc. Further, isolation and biological evaluation of the potential significant compounds are explored for the discovery of drugs as well as to establish the traditional use of the plants.

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