Cytotoxic Evaluation of Antioxidant and Gamma-Amino Butyric Acid (GABA) Enriched Mango (*Mangifera indica cv*. Chaunsa) Mesocarp Extract on HER2-Positive Breast Cancer Cells

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Summary: Mango (*Mangifera indica L*. cv. Chaunsa Sumer bahist) fruit is renowned for its flavor and aroma. Besides, it had been found to have large antioxidant content as well as anti-cancer and anti-diabetic properties. However, experiments to test both the antioxidant content and health benefits are still needed. Here, in the ripe mango mesocarp, we have measured the expression of 11 genes playing a role in flavor and aroma development. An up-regulation in expression of fructose-bisphosphate_aldolase (*MiFBA6)*, glutamate decarboxylase (*MiGAD)*, catalase (*MiCAT1*), glutathione-S-transferase (*MiGSTF6*) and nucleoredoxin (*MiNRX1*) genes was recorded. *MiGAD* gene expression indicated the presence of γ-aminobutyric acid (GABA) metabolite in mesocarp tissue of Chaunsa mango. Metabolite profiling was carried out by gas chromatography-mass spectrometry. A total of five antioxidant compounds with known anticancer activities were characterized in mango mesocarp. It was found metabolites belong to various chemical groups such as: monoterpene alcohols, aroma volatiles, glycoside, monosaccharides, disaccharides, trisaccharides, oligosaccharides, sugar alcohols, sugar acids, essential oils, amino acids, ketone bodies, phytohormones and fatty acids. Seven antioxidant metabolites with known anticancer activities were characterized in mango mesocarp including: 3-hydroxybutyric acid, linalool, geraniol, erythritol, L-(+)-tartaric acid, myo-inositol and lactitol. A total of eight distinct aroma generating siloxanes were characterized in this cultivar. Positive antioxidant activity was also observed in the ethanolic extract of Chaunsa fruit pulp. Chemotherapeutic potential of phenolic compounds found in the ethyl acetate (EtOAc) extract on human breast cancer cell line AU565 was also investigated. The EtOAc extracts effectively suppressed adenocarcinoma growth indicating the anticancer potential of Chaunsa mango fruit.

Keywords: Mango, Anticancer, Antioxidant, Quantitative PCR, GC-MS analysis.

Introduction

Mango (*Mangifera indica L*.) is the second largest fruit crop cultivated after citrus. [1] Mango cv. 'Chaunsa' is the sweetest cultivar of Pakistan with less fiber in pulp. Pakistan is the 6th leading global exporter of mango. The best export quality mango cultivars include Chaunsa, Sindhri, Langra and Anwer ratol. The polyphenols composition and antioxidant potential of 'Chaunsa' peel have already been investigated. [2] The metabolic composition of 'Chaunsa' sap has been studied. [3] However, the metabolome and anticancer potential of post-harvest ripe Chaunsa mesocarp was not characterized before. Flavonoids, [4] phenolic acids [5] and gamma amino butyric acid (GABA) [6] have been reported to induce apoptosis in different cancer cells.

Human breast adenocarcinoma cells have been known to metastasize the brain by adapting to the GABA metabolism as their energy source. [7] Many studies conducted indicated that mango pulp is rich in ascorbic acid, antioxidants, carotenoids, polyphenols and terpenoids. These metabolites inhibit cancer cell proliferation [8] by up-regulating pro-apoptotic biomarkers, cell cycle regulators, cell cycle arrest and lowering reactive oxygen species formation. [9] Mango pulp extracts from 'Keith' mango had chemotherapeutic potential against breast cancer by affecting PI3K/AKT pathway and miR-126. [10] However, the mechanism of antioxidants was not thoroughly examined in these studies.

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Thus in the present study, we aim to characterize the chemotherapeutic agents in mango cv. 'Chaunsa' mesocarp, along with its antioxidant capacity and antiproliferative activity, for which the human breast adenocarcinoma AU565 was utilized in the experiment. Moreover, here we also assesed the chemotherapeutic potential of GABA [11] and γ-Hydroxybutyric acid. [12] Human breast adenocarcinoma cells (AU565, ATCC® CRL-2351™) is characterized as HER2 positive, estrogen receptor (ER) negative with wild type *PIK3CA* mutation, loss in *PTEN* function and shows the high response to drugs: Trastuzumab and Lapatinib. [13]

Plant material collection

Field procedure

Plant material was harvested in June, 2018, in three technical replicates from a single tree at the International Center for Chemical and Biological Sciences, University of Karachi, district Sindh (24.9418° N, 67.1207° E), the mean temperature was 35 ± 2 ^{0} C. The mesocarp tissue sample was taken from artificially ripened fruits (10 days after harvest). The flesh was removed from fruits and immediately frozen in liquid nitrogen and stored at -80 ^{0} C for further experiments.

RNA extraction and cDNA synthesis

Mango mesocarp tissue was ground in liquid nitrogen using a mortar and pestle and 10 mg of sample was prepared for total RNA extraction by following the CTAB method. [14] Total RNA quality was evaluated by measuring 260 nm / 280 nm and 260 nm / 230 nm ratios by using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA integrity was checked using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA). 1 μg-μL of the total RNA amount was calculated by Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Experimental

Selection of genes regulating metabolic biosynthesis pathways in mango cv. Chaunsa

Orthologous gene transcripts catalyzing carbohydrate metabolism (Fructose-bisphosphate_aldolase: *MiFBA6*), shikimate pathway (3-Deoxy-D-arabinoheptulosonate 7-phosphate synthase: *MiDHS1*), flavonoid biosynthesis (Chalcone synthase: *MiCHS1*), monoterpenoid biosynthesis (Linalool synthase: *MiTPS14*) and alanine, aspartate and glutamate metabolism (gamma-aminobutyric acid: *MiGAD*) were selected from the Mango RNA-Seq database of mango exocarp genes including 107,744 annotated unigenes (http://bioinfo.bti.cornell.edu/cgi-bin/mango/index.cgi). [15] Gene-specific primers for quantitative real-time PCR were designed. Genes selected for quantitative real-time PCR are represented in (Table-1).

Table-1: Characterization of genes associated with flavor and aroma metabolism in mango. The Chaunsa mango genes analyzed in this study were named after the most similar *Arabidopsis thaliana* genes. Prefix "*Mi*" is for *Mangifera indica*.

Mango gene ID	Transcript ID (*Mi* ID) Mango RNA-Seq Database	BLASTX Acc. No. (Best hit NCBI ID/ E_value)	Protein identity/ Query cover (%)	Gene product	Physiological role of gene product
MiFBA6	**Fructose-bisphosphate aldolase (MIN045720)**	**XP_020884244.1 (0.0)**	**315/358**	**D-glyceraldehyde 3-phosphate**	**Glycolysis/ Gluconeogenesis pathway**
MiDHS1	**3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase (MIN101907)**	**OAO98609.1 (0.0)**	**423/524**	**3-deoxy-D-arabino-hept-2-ulosonate 7-phosphate**	**Shikimate pathway**
MiCHS1	**Chalcone synthase (MIN008659)**	**AIB06737.1(5e-101)**	**144/144**	**Naringenin Chalcone**	**Flavonoid metabolism**
MiTPS14	**Linalool synthase (MIN034233)**	**BAP75559.1 (0.0)**	**368/574**	**(3S)-linalool**	***Mono-terpenoid* Biosynthesis**
MiGAD1	**Glutamate decarboxylase, putative (MIN030549)**	**NP_197235.1 (0.0)**	**428/503**	**4-aminobutanoate (GABA)**	***Alanine, aspartate and glutamate metabolism***
MiWun1	**Wound-induced protein (MIN012085)**	**EOY17640.1 (4e-76)**	**124/176**	**Cellular senescence pathway regulation protein**	**Cellular senescence**
MiGI	**Gigantea (MIN091141)**	**XP_006473104.1 (0.0)**	**1013/1171**	**Nuclear protein GIGANTEA**	**Plant Circadian rhythm control, photoperiodic flowering and cell differentiation**
MiNRX1	**Nucleoredoxin, putative (MIN030177)**	**XP_006438373.1 (0.0)**	**424/562**	**Probable Nucleoredoxin 1**	**Oxidative stress control**
MiCAT1	**Catalase (MIN013296)**	**XP_006435361.1 (0.0)**	**429/493**	**Catalase**	**Oxidative stress control**
MiPER42	**Peroxidase (MIN049184)**	**XP_006467438 (0.0)**	**276/309**	**Peroxidase 42**	**Oxidative stress control**
MiGSTF6	**Glutathione S-transferase 1 family protein (MIN073310)**	**XP_006444273.1 (6e-119)**	**163/213**	**Glutathione S-transferase F6**	**Detoxification**

Differential gene expression analysis by reverse transcription-quantitative real time PCR (RT-qPCR)

High quality RNA extraction was followed by cDNA synthesis for quantitative PCR by Revert Aid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The reaction mixture contained 5 µL of cDNA (from 20-fold diluted resulting cDNA), 15 µL of SYBR® Green 1 qPCR Master Mix (Promega, Wisconsin, United States), 1 µL (10 µM) of each sense and anti-sense primer, and DEPC-treated water to a final volume of 25 µL per well. Thermal cycling conditions consisted of 10 min at 95 ^{0} C, followed by 40 cycles at 95 ^{0} C for 15 s, 60 ^{0} C for 1 min and 72 ^{0} C for 30 s. Quantitative real-time PCR analysis was performed in a Stratagene Real-Time PCR system (Agilent Technologies, Santa Clara, CA, USA). High-resolution melt curve analysis was used to determine the specificity of the amplicon. The PCR products were also purified, and their concentration was measured to calculate the number of cDNA copies.

The expression level of all genes was calculated by the relative quantification method by using *MiActin*1 (GenBank accession No. JF737036) as a housekeeping gene based on its previous selection as endogenous control gene mango tissue samples. [15]

Extract preparation for antioxidant activity

The extracts were prepared from Mango fruit pulp by using the method described [16] with slight modifications. 100 g of tissue sample was crushed in liquid nitrogen and collected in 15 mL centrifuge tube. 10 mL of acidic ethanol-water (50:50, v/v pH 2) was added and vortexed for 15 min (Vortex IKA MS3) and sonicated (Branson Sonicator) for 30 minutes in cold water. The tube was centrifuged at 6000 RPM (Thermo Fisher Scientific SL16R) for 15 min at 4 ^{0} C and supernatant was recovered. 15 mL of acetone-water (70:30) was added to the residue and sonicated. This was repeated three times. The supernatants were collected after centrifugation. The extracts were stored at -80 ^{0} C.

GC-MS analysis

Freeze dried Chaunsa mango mesocarp tissue samples were extracted for gas chromatography-mass spectrometry (GC-MS) analysis using the method reported by Lim *et al*. [17] using Ribitol (1 mg/mL in water) as an internal standard. Samples were incubated with 50 µL of methoxyamine hydrochloride (10 mg/mL in pyridine) for 1h at 70 ^{0} C. 50 µL of N-methyl-n-(trimethylsilyl) trifluoroacetamide was added to sample followed by incubation for 30 min at 37 ^{0} C followed by centrifugation at 12,000 rpm for 10 min. 100 µL of the supernatant was transferred to autosampler vial (Innotech, Japan). 1 µL of the sample was injected to a gas chromatograph (7890, Agilent Technologies Santa Clara, CA, USA) coupled with to a mass spectrometer (7000, Agilent Technologies, Santa Clara, CA, USA) via an auto sampler in a split mode (split / column flow ratio 10:1). The chromatographic separation was achieved on silica capillary column OPTIMA, 30 m x 0.25 mm i.d. of film thickness 5 - 0.50 µm (MACHEREY-NAGEL, Germany). The pressure of the carrier gas (Helium) was 9.05 pounds per square inch at the initial oven temperature with flow-rate of 3 mL/min. The injector temperature was maintained at 250 ^{0} C; oven temperature was 50 ^{0} C for 2 min initially, rise to 250 ^{0} C at the rate of 10 ^{0} C/ min for a total run time of 80 min. The temperature of the transfer line and of the ion source was set to a value of 320 ^{0} C and 280 ^{0} C. Mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range of 50-700 *m/z*. Data were analyzed by using Mass Hunter software (Agilent) for further processing. compounds. Peak identification of compounds was performed by comparison of obtained mass spectra with those available in the Wiley and NIST 2008 libraries (Wiley Registry TM, 8th Edition Mass Spectral Library, and the NIST 09 Mass Spectral Library [NIST/EPA/NIH] 2008 version), with an acceptance criterion of a score match $\geq$ 70% and the relative percentage contents of all the compounds were calculated by normalizing the peak areas. For functional analysis and metabolic pathways annotation of GC-MS identified volatile compounds, online available MetaboAnalyst tool was used. [18] The data of quantitative real time PCR were analyzed with a variance analysis using a completely randomized design with 95% of statistical significance. Tukey test was utilized when the variance analysis showed statistical differences.

Antioxidant activity analysis by DPPH free radical-scavenging assay

The antiradical activity of mango fruit inflorescence and fruit pulp extracts were determined based on the radical-scavenging effect of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical by performing DPPH assay.

DPPH solution (95 µL, 300 µM) in ethanol was mixed with test solution (prepared extract, 5 µL, 500 µM) in DMSO. The reaction was allowed to

progress for 30 min at 37 ^{0} C in dark. The absorbance was monitored by Tecan (Magellan, Tecan Trading AG, Switzerland) instrument. The absorbance was measured at the following conditions: monochromator light spectrum range 200-1,000 nm for 5 seconds. Upon reduction, the color of the solution fades (violet to pale yellow) at which the percent radical scavenging activity (% RSA) was determined by comparison with a DMSO treated control. The concentration which caused a decrease in the initial DPPH concentration by 50% was defined as IC_{50} values. The reaction was carried out in triplicate.

N-acetyl-L-cysteine and Gallic acid were used as positive controls. The % radical scavenging activities of compounds were calculated by using the following equation:

% RSA of test compound = (1- Absorbance of test sample) / Absorbance of the DMSO treated control * 100

The IC_{50} values for DPPH radical scavenging assay were analyzed by using the EZ-fit enzyme kinetics software program (Perrella Scientific Inc. Amherst, MA, USA) and the data obtained from in vitro experiments were expressed as mean ± standard error.

Cell Lines and Culture

Human breast adenocarcinoma (AU565, ATCC® CRL-2351™) was cultured for treatment and as control. The cell line was purchased from the American Type Culture Collection (ATCC®, Rockville, MD, USA). The cells were cultured in DMEM/5% FBS/penicillin (100 U/mL)/L-arginine + L-asparagine + L-glutamine + sodium pyruvate in 25 cm^2 culture dishes under standard conditions of 5% CO_2, 37 ^{0} C, and 95% relative humidity.

Cell viability assay

DMSO-dissolved Chaunsa mango mesocarp extract was evaluated by the MTT reduction assay following a previously reported procedure. [19] AU565 adenocarcinoma cells were treated with EtOAc mesocarp extract and the viability was determined using MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assays. AU565 cell suspension (50 µg/mL per well) was placed in 96-well Corning Costar® culture plates and incubated for 48 h. The standard was dissolved at a final DMSO concentration (0.25%). AU565 cells were then treated with different concentrations of the extract for 48h. Cells were then washed with DMEM / 5% FBS, followed by subsequent addition of 20 µL of MTT (5mg/mL) and incubated for an additional 3h.

MTT-formazan crystals were formed in metabolically viable cells and were dissolved in 100 µL DMSO, and their spectrophotometric absorbance was measured at a wavelength of 540 nm using a microplate reader (Thermo Scientific Multiskan FC, CA, USA). The results were recorded as IC_{50} values (≤ 5µg/mL).

Results and Discussion

Genes regulating flavor, aroma and flavonoid metabolism in plants

In climacteric fruits, reactive oxygen species homeostasis regulates fruit development and initiates ripening. [20] Increase in the damaging reactive oxygen species production such as superoxide anion and hydrogen peroxide, may have triggered the higher expression levels of flavor and pigment regulating genes *MiFBA6*, *MiGAD* and *MiCHS1* at post-harvest ripened stage of Chaunsa mango fruit. Significant down-regulation was observed for *MiTPS14* gene for Linalool biosynthesis (Fig. 1a).

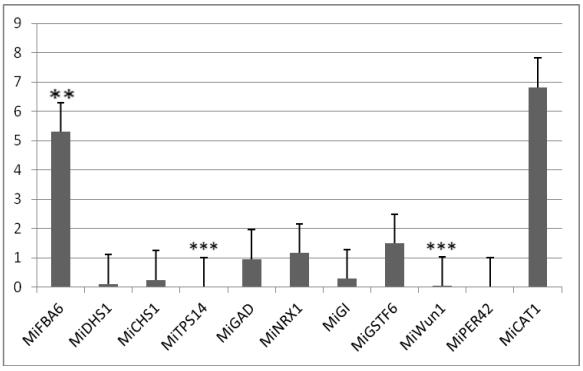


Fig. a: Expression of genes regulating glycolysis and gluconeogenesis (*MiFBA6*, Fructose-bisphosphate aldolase) shikimate pathway (*MiDHS1*, 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase), flavonoid biosynthesis pathway (*MiCHS1*, Chalcone synthase), terpenoid biosynthesis pathway (*MiTPS14*, Linalool synthase) and Alanine, aspartate and glutamate metabolism (*MiGAD*, gamma-aminobutyric acid) was determined in Chaunsa mango fruit at 10 days of harvest (DAH). Statistical significance in gene expression is indicated by asterisks. Asterisks represent the statistical significance at p-value ≤ 0.05 (*), p-value ≤ 0.005 (**) and p-value ≤ 0.0005 (***).

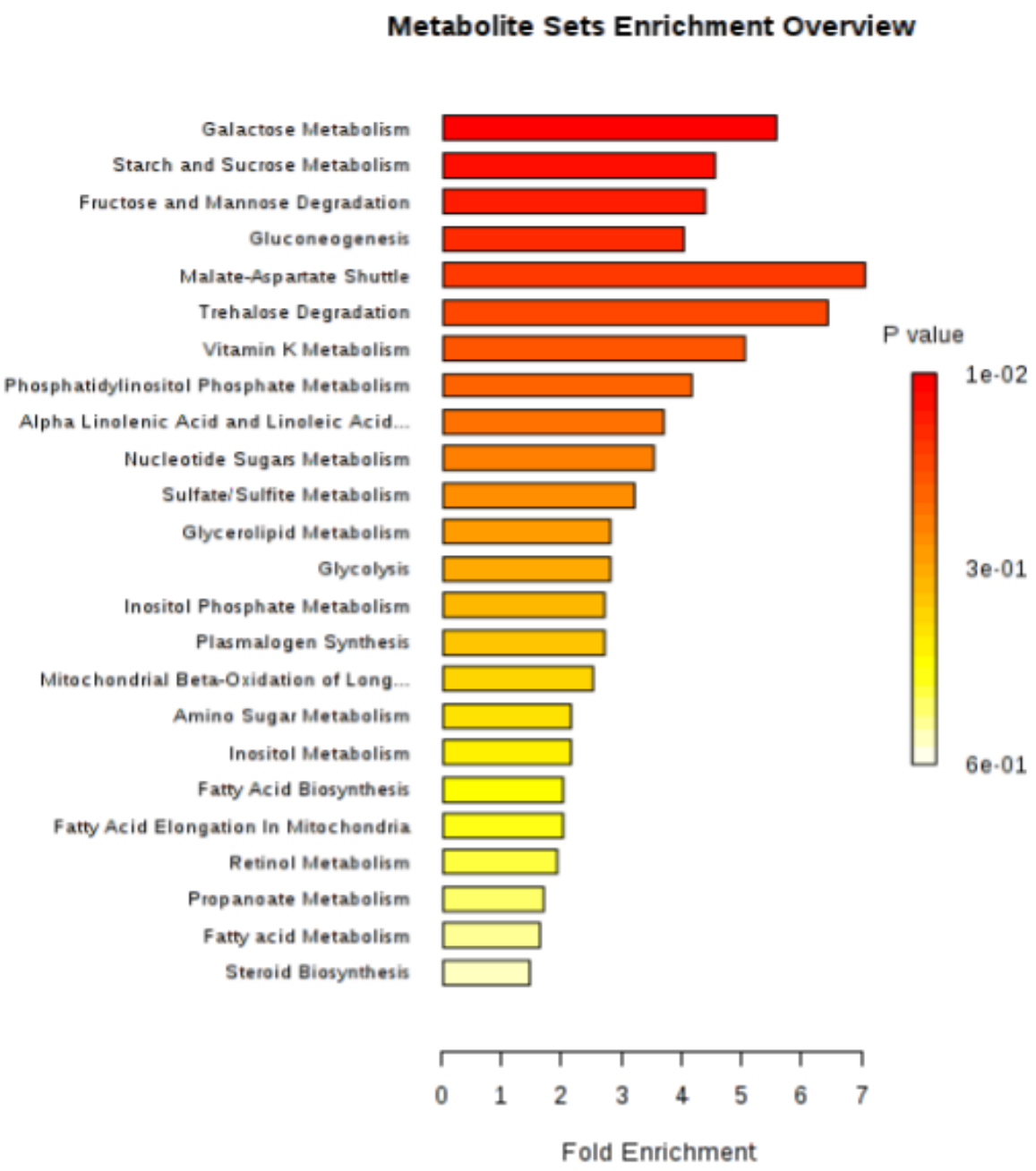


Fig_2a: Representative metabolic pathways characterized in Chaunsa mango mesocarp.

In plants, Fructose 1,6 bisphosphate_aldolase (FBA) not only regulates glycolysis, gluconeogenesis and Calvin cycle, but it is also involved in biotic and abiotic stress responses. [21] In plants, during carbon fixation and sucrose metabolism, Fructose 1,6 bisphosphate_aldolase (FBA) reversibly cleaves fructose-1,6- bisphosphate (FBP) into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). A significant increase in expression of *MiFBA6* indicates an increase in reactive oxygen species and mango fruit's energy demands. Interestingly, starch and sucrose metabolic pathway were also found to be up-regulated in 'Chaunsa' mango mesocarp metabolic pathways characterization by GC-MS analysis (Fig. 2a). Moreover, Fructose is the major soluble sugar whose concentration increases at ripened stage in fruit. [22] Accumulation of fructose may have significantly increased *MiFBA6* expression in 'Chaunsa' mesocarp. Fructose and Mannose degradation pathway was also found to be up-regulated in metabolic pathway analysis (Fig. 2a).

Table-2: Identification of antioxidants by GC/MS chromatography analysis.

Compound name	Retention time (minutes)	Molecular weight	Molecular formula	References
3-Hydroxybutyric acid	13.98	248	$C_{10}H_{24}O_3$	(Pinto, Bonucci *et al.* 2018)
Linalool	15.44	226	$C_{13}H_{26}O$	(Shen, Wang *et al.* 2013)
Geraniol	17.70	226	$C_{13}H_{26}O$	(ChO, So *et al.* 2016)
Erythritol	20.566	410	$C_{16}H_{42}O_4$	(Regnat, Mach *et al.* 2018)
L-(+)-Tartaric acid	23.249	438	$C_{16}H_{38}O_6$	(Yang, Chang *et al.* 2011)
Myo-Inositol	47.94	612	$C_{24}H_{60}O_6$	(Bizzarri, Dinicola *et al.* 2018)
Lactitol	52.51	344	$C_{12}H_{24}O_{11}$	(Geen, Evans *et al.* 1995)

In transgenic *Arabidopsis thaliana*, chalcone synthase gene from *Abelmo schusesculentus* was found to be up-regulated under abiotic stress. In transgenic *Ae*CHS gene at reduced reactive oxygen species by enhancing flavonoid accumulation and increasing the expression of flavonoid biosynthesis pathway related genes. [23] An increase *in MiCHS1* expression in 'Chaunsa' mango mesocarp indicates the accumulation of various flavonoids which were characterized by GC-MS analysis and their high antioxidant potential was further assessed by DPPH free radical-scavenging assay (Table-2 and Supplementary S1).

In tomato fruit (*Solanum lycopersicum L.*), five glutamate decarboxylase (*GAD*) genes regulate γ-aminobutyric acid (GABA) biosynthesis. A moderate *SiGAD* gene expression is reported in tomato fruit at red fully ripened stage. [24] In our study, we observed increased expression of *MiGAD* gene, indicating enhanced flavor attributes.

Metabolic profiles using GC-MS analysis

GC-MS analysis of EtOAc extracts of Chaunsa mesocarp was carried out. A total of 101 metabolites regulating various biological pathways (Fig. 2a) were characterized in post-harvest ripe Chaunsa mango fruit. These putative metabolites were belong to various classes of compounds including monoterpene alcohols, aroma volatiles, glycoside, monosaccharides, disaccharides, trisaccharides, oligosaccharides, sugar alcohols, sugar acids, essential oils, amino acids, ketone bodies, phytohormones and fatty acids. Representative chromatogram indicating Ribitol as an internal standard (Fig. 2c). The EtOAc extracts included several phenolic acids and sugar acids including inosine, 3-oxovaleric acid, quinic acid, shikimic acid, 1H-indole-3-acetic acid, gallic acid, D- glucuronic acid, gluconic acid, hexadecanoic acid, 11-cis-octadecenoic acid, α-D-glucopyranosiduronic acid, myristic acid, hexadecanoic acid, propanoic acid, acetic acid, erythro-pentonic acid, glyceric acid, D-(+)-malic acid, hexanedioic acid, L-threonic acid, L-(+)-tartaric acid, 2,3,4,5-tetrahydroxypentanoic acid and galacturonic acid.

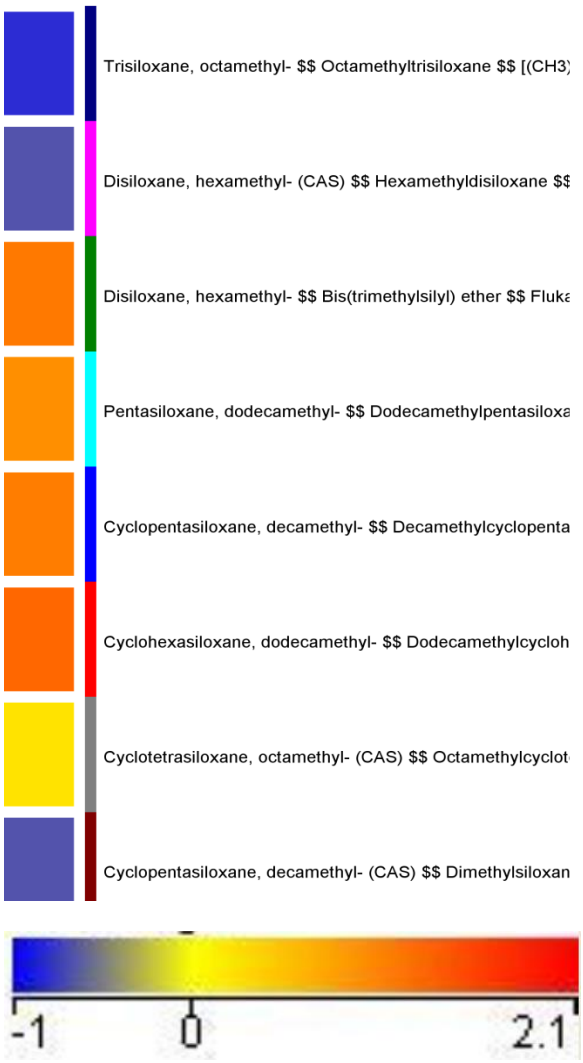
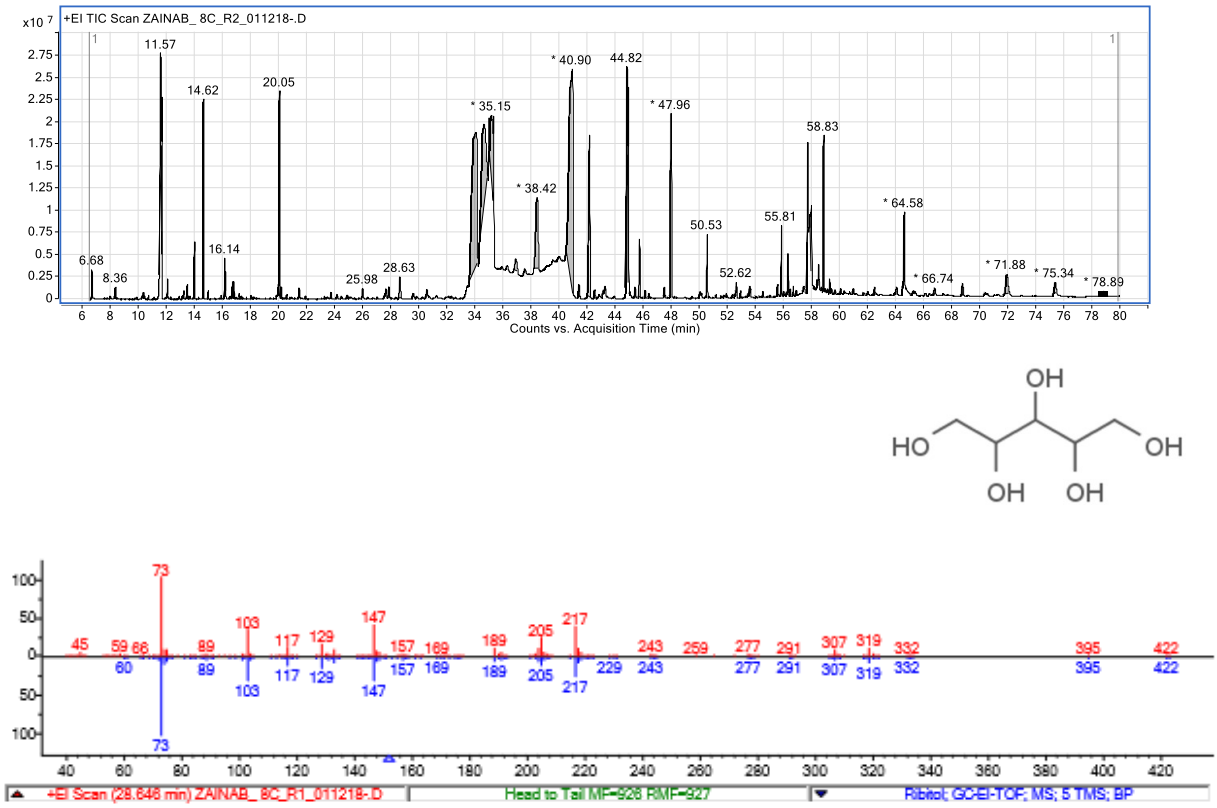


Fig. 2b: Qualitative abundance of aroma volatiles in artificially ripened Chaunsa mango fruit.

It is known that degradation products of aldehydes like alcohols and esters of 4-hydroxy acids called lactones confer specific aroma and flavor to fruits and vegetables. [25] In this study we have identified 2 lactones: 2,3,4,5-tetrahydroxypentanoic acid-1,4-lactone and erythronic acid- γ-lactone. We have identified 8 sugar alcohols: L-iditol, Erythritol, 1,5-anhydro-D-glucitol, D-arabitol, Myo-inositol, Glycerol, D-glucitol and Pentitol.



Name: Ribitol; GC-EI-TOF; MS; 5 TMS; BP
Formula: $C_5H_{12}O_5$
MW: 152 CAS#: 488-81-3 ID#: 42437 DB: mass bank of europe
Other DBs: None
Comment: RetentionTime=416.034, RetentionIndex=1710.9
10 largest peaks:
73 999 | 147 292 | 103 281 | 217 235 | 129 127 |
205 99 | 117 94 | 74 85 | 133 59 | 218 57 |
Synonyms:
no synonyms.

Fig. 2c: Antioxidant contents of EtOAc extracts of 'Chaunsa' were analyzed using GC–MS. Representative GC–MS chromatogram of volatiles separated from EtOAc extracts of Chaunsa mango pulp with representative total ion mass spectrum of Ribitol (recorded at retention time, 416.034 min) from 'Chaunsa'.

A total of 46 unique volatile metabolites were characterized in Chaunsa mango cultivar. These volatile compounds characterized were organic acids, sugar and fatty alcohols, lactones, furanones, monoterpenoids, sesquiterpenes, aldehydes, ketones, fatty acids and phenols. These metabolites mostly impart fruity, minty and floral notes.

Hexamethyldisiloxane, Dodecamethyl-pentasiloxane, Decamethylcyclopentasiloxane and Dodecamethylcyclohexasiloxane were the most qualitatively abundant aroma volatiles amongst the identified volatiles (Fig. 2b). Furanone is a heterocyclic organic compound that gives pineapple-like flavor and aroma to different mango cultivars. We have identified Cis-3,4-dimethyldihydrofuran-2(3H)-one and Trans-3,4-dimethyldihydrofuran-2(3H)-one. Geraniol and Linalool were the unique monoterpenoids identified in Chaunsa mango fruit imparting sweet floral notes to this cultivar. (R)-3-hydroxybutyric acid was characterized as the only ketone body in mango pulp. It imparts fruity, winey grape-like aroma in apples and grapes. [26]

Other volatile metabolites identified were 1-(3-methylbutyl)-2,3,4,6-tetramethylbenzene, 2,2,4,4,8,8-Hexamethyl-6-methylene-3,7-dioxa-2,8-disilanonane, bis(Trimethylsilyl)-2-

[(trimethylsilyl)oxy]pent-2-enedioate, 2,2,4,4,7,7-Hexamethyl-3,6-dioxa-2,7-disilaoctane, (3R)-3-Phenyl-2,3-dihydro-1H-isoindol-1-one, 4-Methyl-4-[(trimethylsilyl)oxy]-2-pentanone, 2-Methyl-4-Keto-Pentan-2-ol, 4-Methyl-4-[(trimethylsilyl)oxy]-2-pentanone, Propionic acid, N-(Trimethylsilyl)furan-2-carboxaldimine, Butanoic acid, 4-Oxo-4H-[1]benzofuro[3,2-b]thiopyran-3-carbaldoxime, Silanol, 1,2,3-Butantriol, Succinic acid, Erythro-Pentonic acid, Glyceric acid, D-(+)-Malic acid, 1-(1-methyl-2,2-D2-2-)-4-(2-methyl-2-)benzene, Hexanedioic acid, 3,4-dihydro-7-isopropoxy-5,9,10-trimethoxy-r-1,c-3-dimethyl-1H-naphtho[2,3-c]pyran, 3-Oxovaleric acid, Uridine 5'-diphospho-N-acetylglucosamine, Shikimic acid, Quinic acid, Gallic acid, D-Glucuronic acid, Gluconic acid, Galacturonic acid, Hexadecanoic acid, α-D-Glucopyranosiduronic acid, Lactitol, Inosine, 1,2,3,4,5-Pentaisopropylbis(cyclopentadienyl) cobalticimium and 1,1-Disiloxanediamine. These metabolites may be considered as biomarkers in cultivar selection and as a harvesting index for mango fruits. [27]

Two saturated fatty acids: 2-Monopalmitin and Myristic acid, 1 sugar acid: Galactonic acid and 1 sugar alcohol: Pentitol, 2-Monopalmitin (Palmitic acid) were also characterized in ripe Chaunsa mango mesocarp. These may be playing a role as an intermediate in secondary metabolite biosynthesis. Myristic acid characterized at this stage indicated the biosynthesis of different fatty acids. [28] Galactonic acid identified at this stage indicated the galacturonatereductase and aldonolactonase activities which increase the ascorbate (Vitamin C) level thereby regulating detoxification of ROS. [29]

We also characterized the metabolic pathways in post-harvest Chaunsa mango fruit mesocarp (10 DAH) which indicated that the highest number of metabolites is part of the galactose metabolism followed by starch and sucrose metabolism, fructose and mannose degradation and gluconeogenesis (Fig. 2a).

Antioxidant activity analysis

The antioxidant activity of the mango extracts was examined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Good antioxidant activity was observed in ripe Chaunsa mango mesocarp. Chaunsa fruit's leaves, peels, stems and kernel also exhibit good antioxidant potential. [30] Five metabolites with antioxidant and anticancer potential were characterized in mesocarp tissue including: L-(+)-Tartaric acid, Myo-inositol, Geraniol, Linalool, Lactitol and Erythritol (Table-2).

The IC_{50} ± SEM (µM) value of the acidic ethanol-acetone extracts for DPPH was 0.0185±0.0008 mg/mL for Chaunsa mango pulp (10 DAH). IC_{50} ± SEM (µM) values of the standard Gallic acid was 0.0044±0.0003 mg/mL and that of N-acetyl-L-Cysteine was 0.020±0.003 mg/mL.

Anti-proliferative activity against AU565 adenocarcinoma cells

EtOAc extracts from mango *cv.* chaunsa mesocarp induced apoptosis in AU565 breast cancer cells. Human breast adenocarcinoma AU565 cells were treated with EtOAc extracts for 48 h , the extracts showed best anti-proliferative activity (AU565; IC_{50} (µg/mL)).

Traditional medicines use plant extracts which are rich in polyphenols for various ailments. Mango *cv.* Ataulfo peel extracts showed significant anticancer activity against the human colon adenocarcinoma LS180 cells. [5] While phenolic and flavonoid rich EtOAc extracts of *Avicennia marina* leaves also showed highest anticancer activity against different human breast cancer cell lines (AU565, MDA-MB-231, and BT483) and human liver cancer cell lines (HepG2 and Huh7). [31]

Conclusion

Several metabolic pathways are part of the Chaunsa mango fruit. The genes regulating carbohydrate metabolism, aroma, flavor and flavonoid biosynthesis seems to be involved in 'Chaunsa' mango fruit ripening were characterized. The presence of antioxidant compounds in mesocarp tissue inhibited AU565 adenocarcinoma cell proliferation. It can be suggested that Mango (*Mangifera indica L.*) cv. 'Chaunsa' is one of the most important Pakistani mango cultivars of nutraceutical interest.

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