Metabolites profiling of Sapota fruit pulp *via* a multiplex approach of GC/MS and UPLC/MS in relation to its lipase and α-glucosidase inhibition effects

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Abstract:

Background. Sapota fruits, *Manilkara zapota* L., are juicy, and nutrient-rich fruits aside from their several health benefits.

Methods. The current study presented anintegrated metabolomic profiling of sapota fruits pulp*via*GC/MS and UPLC/MS, quantification of total phenolics and flavonoids, antioxidant capacity, and inhibitory effect of pancreatic lipase (PL) and *α*-glucosidase enzyme inhibitions.

Results.GC/MS analysis of silylated primary polar metabolites led to the identification of 68 compounds belonging to sugars (74%), sugar acids (18.3%), and sugar alcohols (7%) mediating the fruit sweetness. Headspace SPME-GC/MS analysis led to the detection of 17 volatile compoundsbelonging to nitrogenous compounds (72%), ethers (7.8%), terpenes (7.6%), and aldehydes (5.8%). Non polar metabolites profiling *by*~~*via*~~ HR-UPLC/MS/MS-based GNPS molecular networking led to the assignment of 31 peaks, with several novel sphingolipids and fatty acyl amides reported for the first time. The total phenolic content was estimated at 6.79±0.12 mg GAE/g, concurrent with antioxidant capacities of the fruit at 1.62 ±0.2, 1.49±0.11, and 3.58±0.14 mg TE/g *via* DPPH, ABTS, and FRAP assays, respectively. *In vitro* enzyme inhibition assays revealed considerable PL inhibition activity (IC_{50} = 2.2 ± 0.25 mg/mL), whereas no inhibitory effect towards *α*-glucosidase enzyme was detected. This study provides deep insight into sapota fruit's flavor, nutritional attributes, secondary metabolites, and its medicinal~~biological~~ effects.

Keywords: *Manilkara zapota* L.; SPME-GC/MS; UPLC/MS;GNPS Molecular Networking; lipase inhibitor;*α*-glucosidase inhibitor; antioxidant.

1. Introduction

The enormous diversity of fruits worldwide makes them a potential source for a wide range of nutraceuticals that are useful as edible food and health promoting agents. According to studies,

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Comment [WU5]: Full name, pancreatic lipase

regular consumption of vegetables and fruits can aid in lowering the susceptibility to risky diseases (Bazzano et al., 2003).

Sapotaceae is a family of tropical, evergreen trees and shrubs that comprises more than 50 genera and 1100 species. Their trees are famous for producing latex, gums, timbers from the trunks, oil from the seeds, and most of which produce edible flowers and sweet fruits (Vaghani, 2003). Fruits have been valued for their rich nutritional quality due to their carbohydrates, vitamins, minerals, and fiber content, aside from their multitude health benefits owing to their remarkable bioactive substances. The family is rich in pentacyclic, triterpenoid saponins and their glycosides, which are thought to be related to folk uses of the family as antimicrobial, antitumor, and anti-inflammatory (Baky et al., 2022).

One of the most famous genera in the Sapotaceae family is *Manilkara*, with *ca*. 80 species. The trees of *Manilkara zapota* (L.) Van Royen is the most extensively grown species, native to Central America, especially Mexico and the Caribbean. The long-lived trees are now broadly cultivated in many tropical Asian countries. *M. zapota* trees are recognized for their wood and latex, in addition as a source of sweet, edible fruits. The name "*Manilkara zapota*" has numerous synonyms mentioned by the plant list including *M. zapotilla* (Jacq.), *M. achras* (Mill.) Fosberg, *Achras zapota* or (*sapota*) L., *A. zapotilla* (Jacq.) Nutt., or *Sapota zapotilla* (Jacq.) (The Plant List (2010) , Madani et al., 2018).

M. zapota (L.) fruits, also known as Sapota, Sapodilla, and chicozapote, comprise a wide array of nutrients, minerals, and polyphenols with~~, and hence~~ diverse biological activities. Fruits are uniquely delicious, with a delicate, grainy feel and pleasant smell, covered by thin, yellowish-brown peel (Siddiqui et al., 2014). The nutritional value of fruits is attributed to their high content of carbohydrates 20%, dietary fiber5 %, with small amounts of proteins and fats, in addition to considerable amounts of vitamins A and C. Fruits are rich in minerals especially calcium and potassium, alongside magnesium, sodium, phosphorus, and a lesser amount of iron (Singh et al., 2021, Rivas-Gastelum et al., 2023). Unripe fruits are astringent in taste due to high levels of catechins, gallic acid, chlorogenic acid, gallotannins, and proanthocyanidins (Ma et al., 2003). Upon maturation, free sugars increase concurrently with a drop in phenolics. Aside from phenolics, fruits are also rich in triterpenoids represented by β-amyrin-3-(3'-dimethyl) butyrate and lupeol-3-acetate (Fayek et al., 2013). In a comparative study among the different *M. zapota* (L.) parts, the highest level of phenolic acids and flavonoids was found in leaf, followed by seed,

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peel, and flesh. Consequently, leaves showed the most pronounced~~potent~~ antioxidant effect using *β*-carotene bleaching and DPPH*in vitro* assays (Tamsir et al., 2020).Such promising antioxidant actions of the fruit, along with the lipoidal content of oleic, linoleic acids, and glycerol, make the fruits appropriate for dermatological formulations for anti-wrinkles, anti-aging medications (Shafii et al., 2017). ~~Other effects reported in fruit include~~ The ~~its~~ aqueous extract reported to exhibit potent anti-hypercholesterolemic, antihyperglycemic, and antioxidant activities (Fayek et al., 2013). Both leaf and fruit extracts showed a strong influence on lowering sugar and cholesterol levels in blood ~~levels~~. Moreover, fruits revealed a decrease in body weight, posing it suitable for~~in~~ obesity and diabetes management (Barbalho et al., 2015).

Suppressing pancreatic lipase enzyme helps in the treatment of several metabolic disorders, including diabetes, hyperlipidemia and obesity which exerts severe health issues for all body organs, especially in developing countries(Lunagariya et al., 2014).

The current study presents a multiplex approach employing Gas Chromatography coupled with Mass Spectrometry(GC/MS), and High-ResolutionUltra-high performance liquid chromatography coupled with tandem Mass Spectrometry (HR-UPLC/MS/MS) for profiling *M. zapota* (L.)fruitpulp targeting its aroma, non volatile polar and non polar metabolites to account for fruit sensory, nutritional, and health attributes. The aroma profile was assessed using solid phase micro-extraction (SPME), whereas primary polar metabolites viz. sugars were analyzed using GC/MS post-silylation. For large molecular weight non polar metabolites analysis, HR-UPLC/MS/MS was employed aided by Global Natural Products Social (GNPS) molecular networking to aid in metabolites identification. In addition, total phenolics and flavonoids were determined for standardization, alongside lipase and *α*-glucosidase inhibition activities of fruit extract.

2. Material and Methods

2.1. Plant Material and Extraction Process

The fresh fruitpulp of sapota (*Manilkara zapota* L.) was collected from Haryana Agriculture University, Hisar, India in December 2022 and was identified by Dr. Rupesh Deshmuk, Central University of Haryana, India. Fruits were immediately lyophilized, peeled and the pulp was taken and ground in liquid nitrogen using mortar and pestle, and stored in closed, air tight bags ~~till further analysis~~ at -20 °C till further analysis. The extraction process was carried out following

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the procedure previously mentioned in (El-Akad et al., 2023). Using a homogenizer (Ultra-Turrax, IKA,Staufen, Germany) at 11,000 rpm, 5 X 60 s with 1 min break intervals, about 150 g of the crushed sample was mixed with 6 mL methanol containing 10 µ~~u~~g/mL umbelliferone (Sigma-Aldrich,St. Louis, MO, USA, purity ≥ 98%) that used as an internal standard and for MS calibration. Further processing, extract was centrifuged at 3000~~x~~×*g* for 30 min after being vortexed for 1 min., ~~then~~ filtered through a 22 m poresize filter and directly used for HR-UPLC-MS/MS analysis.For GC/MS, 100 ul was aliquoted in a glass vial and left to evaporate till dryness under a nitrogen stream. For bioassay, fruit pulp was extracted using 100% MeOH till exhaustion and evaporated using rotavap under vacuum to yield dried yellowish residue stored at -20 °C till further assays.

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2.2. Chemicals and Fibers

The stableflexfiber used forsolid phase micro-extraction (SPME) was covered by divinylbenzene/carboxen/polydimethylsiloxane(DVB/CAR/PDMS, 50/30 µm) and was obtained from Supelco (Oakville, ON, Canada). Chemicals were acquired from Sigma Aldrich (St. Louis, MO, USA). Milli-Q water and solvents that were used for HR-UPLC/MS/MS analysis; formic acid and acetonitrile were of LC-MS grade and obtained from J. T. Baker (The Netherlands).

ABTS [2,20′azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] ≥ 98% purity, DPPH (2,2-diphenyl-1-picrylhydrazyl), ferric chloride for FRAP (ferric reducing antioxidant power), Trolox (6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid; ≥97% purity), Porcine pancreatic lipase (PL) enzyme type II, intestinal *α*-glucosidase, Orlistat, Acarbose from Sigma Aldrich Chemie GmbH (St. Louis, MO).

2.3. GC/MS Analysis of Silylated Primary Polar Metabolites of *M. zapota*FruitPulp:

Analysis of primary metabolites in fruit pulp followed the exact procedure by (El-Akad et al., 2023), in triplicates under the same conditions. The derivatization process was compiled as follows; the dried methanol extract of fruits ~~prepared as in section 2.1.~~ was derivatized using a silylating agent; N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) (150 µL equally diluted with anhydrous pyridine), incubated in an oven for 45 min at 60 °C (Yamato Scientific DGS400 Oven, QTE TECHNOLOGIES, Hanoi, Vietnam), just prior to GC/MS analysis. Silylated compounds were separated on a column 30 m. x 0.25-mm id x 0.25-m film (Rtx-5MS Restek, Bellefonte, PA, USA), and were analyzed under conditions described in (Farag et al.,

2022).Analysis was done in triplicate under the same conditions along with a blank sample to assess for biological variance.

2.4. SPME Analysis of Volatiles in *M. zapota* FruitPulp:

Preparation and investigation of aroma profile in fruit pulp were performed following the same conditions reported in (Farag et al., 2022). A quadrupole mass spectrometer connected to an Agilent 5977B GC/MSD (Santa Clara, CA, USA) was used fitted witha DB-5 column (Supelco, Bellefonte, PA, USA) 30 m x 0.25 mm i.d. x 0.25m film thickness. The scan range of the MS spectrometer was adjusted at *m/z* 40–500 and EI mode at 70 eV. Analysis was done in triplicate under the same conditions along with a blank sample to assess for biological variance.

2.5. Identification of Volatile and Non-Volatile Silylated ComponentsUsing GC/MS

Deconvolution of the GC/MS spectrum was first applied using AMDIS software (www.amdis.net). Detection of compounds was achieved by matching the retention indices (RI) of the detected peaks with those of the n-alkanes series (C8-C30), along with matching their mass spectra with respected databases; NIST011 and WILEY libraries, and standards whenever available.

2.6.High-resolution Ultrahigh-Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry (HR-UPLC/MS/MS)Analysis of Non Polar Metabolites

HR-UPLC/MS/MS analysis was performed using an ACQUITY UPLC system (Waters,Milford, MA, USA) coupled with an HSS T3 column (100 x 1.0 mm, particle size 1.8 µm;Waters). The analysis was accomplished following the precise guidelines as reported by (Hegazi et al., 2022). The tentative identification of compounds was based on the generated molecular formula at an error of 10 ppm or less, and by comparing MS^2 fragments with those reported in literature.

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2.7. Molecular Networking and Metabolites' Annotation of HR-UPLC/MS/MS Data

The HR-UPLC/MS/MS data (acquired in positive ion mode) from the fruit extract was used to create molecular network (MN) using GNPS website (http://gnps.ucsd.edu). The raw data underwent conversion to an open-source format (.mzML) using the MSConvert package (Proteowizard Software Foundation, Version 3.0.19330, USA). The transformed (.mzML) files were then uploaded to the GNPS platform using WinSCP (SFTP, FTP, WebDAV, and SCP client). GNPS parameters included fragment ion tolerance (0.5 u), minimum-matched fragments (4 ions),

minimum pairs cosine score (0.65), and parent mass tolerance (1.0 u), which were used to generate consensus spectra.~~To access the generated molecular network, follow this link (https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=4b2bc3c320234c6e804955a6119d1240)~~

The spectral network was visualized with the aid of Cytoscape 3.9.1. Each spectrum was represented as a node in the visualization, with spectrum-to-spectrum connections forming edges based on structural correspondence identified through MS analysis(Xu et al., 2021,Zia-ur-Rehman et al., 2022)

For natural products dereplication, various databases were searched, including PubChem (https://pubchem.ncbi.nlm.nih.gov/), Metabolome Database (https://hmdb.ca/), Online lipid calculator database (http://www.mslipidomics.info/lipid-calc/), and LIPID MAPS (https://www.lipidmaps.org/).

For visualization of metabolite classes with sapota fruit, acquired tandem mass spectrometry data, and molecular networks were (MNS) constructed. Mass spectrometric data were classified according to the spectra resemblances in the fragmented ions(Ragheb et al., 2023), and to aid in the identification of unknown peaks.

2.8. Total Phenolic (TP) and Total Flavonoid (TF) Contents Estimation

The evaluation of TP content was based on the Folin-Ciocalteu method, previously described by (Babotă et al., 2018). The result was represented as milligrams of gallic acid equivalent per gram sample (mg GAE/g), after triple measurements. For TF content, aluminum chloride assay was used, with results expressed as milligrams of rutin equivalent per gram sample (mg RE/g) (Babotă et al., 2018). Fruit pulp methanol extract for both tests were analyzed, after being re-dissolved and diluted, in 96-well plates using a SPECTROstar® Nano Multi-Detection Microplate Reader (BMG Labtech, Ortenberg, Germany).

2.9. *In vitro* Antioxidant Assays

Two assaysdepending on the free radical scavenging actions; DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS [2,2′-azino-bis(3-ethylbenzothiazoline) 6-sulfonic acid], along with the FRAP (Ferric reducing antioxidant power) technique for ferric reducing capacity, were applied following the protocols of(Babotă et al., 2018). The resulting data were reported as mg of Trolox equivalents per gram sample (mg TE/g) in each case.

2.10. *In vitro* Enzyme Inhibition Assays

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Pancreatic Lipase (PL) inhibition activity was measured colorimetrically based on *p*-nitrophenol release (at 410 nm compared to a blank of denatured enzyme), following a modified method mentioned by (Bustanji et al., 2011). The experiment was run in triplicate and percentage inhibition represented the average of three observations using two concentrations of the extract, expressed in terms of IC_{50} (Half-maximal inhibitory concentration).Orlistat was used as a positive control as a standard PL inhibitor.

Whereas,*α*-Glucosidase inhibitory action was measured following the previously reported~~same~~ protocol(Tanase et al., 2019). The color formed due to *p*-nitrophenol was measured at 405 nm, and acarbose was used as a positive control. The result was calculated as the concentration that inhibited 50% of the enzyme (IC_{50}), after three measurements using two concentrations of the extract.

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3. Results & Discussion

3.1. Metabolites profiling of silylated primary polar metabolites in *M. zapota*fruit pulpas analyzed *via*GC/MS

GC/MS analysis of the non-volatile primary metabolites in fruit was carried out post-silylation to present a comprehensive overview of metabolites (Figure 1), and further account for nutritional and sensory attributesin fruits. As listed in Table (1), 68 compounds were detected belonging to 11 chemical classes. The most abundant metabolite classes as typical in fruits included sugars, sugar acids, and sugar alcohols detected at 74.0, 18.3, and 7 %, respectively. Other detected primary metabolites though at much lower levels included fatty acids/esters (0.22%), organic acids (0.22%), and inorganic acids (0.124%), along with traces of alcohols, terpenes, and nitrogenous compounds.

The high content of sugars in fruit imparts a sweet taste and calories as typical in most fruits.As represented in the TIC (Figure 2), sugars represented major primary metabolites detected in 22 peaks, especially mono-sugars to account for 75% of identified sugars. The most prominent forms included fructose (22.1%), D-glucose (16.6%), and mannose (16.5%). Sucrose (17.9%, peak 63) was the predominant disaccharide.Previous reports on sapota fruit for total sugars fruits revealed that they amountedfor 46 to 52.2% ofits weight (Swami, 2018).

The high level of sugar acids represented by keto-D-gluconic acid (9.8 %) and L-gluconic acidlactone (7.9%) imparts a slightly tangy and acidic taste, which might provide a balanced sensation alongside the intense sweet taste (Karaffa & Kubicek, 2021).

Sugar alcohols with lower calorie intake than free sugars were represented by 5-deoxy-myo-inositol (6.7%). In addition to its low-calorie level, it reduces the body's resistance to insulin and aids in diabetes management (Corrado & Santamaria, 2015).

Although organic acids were present at minor levels (0.22%), they were represented by 13 compounds, with oxalic and pyruvic acidsas major forms.

Fatty acids/esters composition plays a role in nutrition and flavorin fruits, detected at 0.23% including glycerol monostearate, the monoglyceride ester with a sweet taste. Likewise, saturated fatty acid palmitic acid (0.045%) and its monoester, monopalmitin (0.054%), were detected suggesting that fruit is richer in saturated fatty acids.To the best of our knowledge, this is the first detailed report on primary metabolites composition in sapota fruit to account for its nutritive value.

3.2. Aroma Profiling of *M. zapota*FruitsPulp*via* SPME Coupled to GC/MS:

SPME-GC/MS analysis of aroma composition in *M. zapota*fruit revealed the detection of 17 compounds belonging to 8 chemical classes mostly dominated by nitrogenous compounds amountingfor 71.7%.Other classes includedethers (7.8%), terpenes (7.6%), and aldehydes (5.8%) as represented in Figure (2) and Table (2).

The identified nitrogenous compounds (peaks 3, 5, 10 and 11) were detected for the first time in the fruit belonging to isothiocyanates, a hydrolysis product of glucosinolates. The major compound was 3-butenyl isothiocyanate (64.3%),alongsideallyl isothiocyanate (2.8 %).The presence of ethers in fruit provides specific fragrances, represented here by benzyl isoeugenol ether (4.2%), peak 17, in addition to pentyl allyl ether (3.1%), peak 4, and cineole, peak 8 (Kirsch & Buettner, 2013).

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As typical in fruit aroma, a considerable amount of mono- and sesquiterpenes were detected amounting (7.6%) of total aroma composition, withβ-caryophyllene (5.42%), peak 16, and limonene (1.54%) peak 7 as major components. β-Caryophyllene was previously detected insapota fruit volatiles using steam distillation (Pino et al., 2003).Aldehydes, which accounted

for 5.8% of the total fruit aroma, likely contribute to the fruit scent and likewise, protect against their deterioration due to potential antibacterial action (Aljaafari et al., 2022). The major form was hexanal at 4% to impart an apple-like odor (Plotto et al., 2017), alongside benzyl alcohol (2.6%) and with light fragrant smell (Kulkarni & Mehendale, 2005) and all to contribute to sapota fruit specific scent.

3.3. Non polar Metabolites Profiling of *M. zapota* Fruit as Analyzed *via* HR-UPLC/MS/MS

Considering that GC/MS can only detect low molecular weight polar phytochemicals in food, and to provide comprehensive composition of sapota fruit metabolome,HR-UPLC/MS/MSwas employedto complement GC/MS and target large molecular weight lipids(Islam et al., 2021). Herein, a list of tentatively identified metabolitesof *M. zapota*fruit is presented in Table (3), along with their chromatographic and spectroscopic data (Figure 3). Major identified metabolites containedbelonged to lipoidal components e.g. fatty acyl amides, phospholipids,and sphingolipids, and contrary to low levels of lipids detected using GC/MS more suited for polar chemicals profiling. Other classes detected at minor levels includedfatty acyl esters, nitrogenous compounds, glycol, amino acids and diethanolamines. To aid in metabolites assignment, molecular networking was used for HR-UPLC/MS/MS dataset visualization. The MN afforded a total of 346 nodes, of which 141 clustered nodes and 205 self-looped nodes were detected (Figure 4). The visual aid of MNS showed the diverse metabolite classes, which assisted in analogs identification. The substantial clusters of positive MNbelonged to oxylipids including cluster A (sphingosine and sphinganine), cluster B (fatty acyl amides), cluster C (phytosphingosine), and cluster D (fatty acyl esters), (Fig. 4).

3.3.1. Identification of fatty acyl amides

Fatty acyl amides, a subclass of lipids,exist as bioregulators for lipids in plants and are formed through amidation of fatty acids (Tanvir et al., 2018). Seven fatty acyl amides were identified in sapota fruitextract based on neutral losses of 14 amu, indicative of an acyl group(Suppl. Fig. S1). Further, the annotation of saturated fatty acyl amides$[M + H]^+$ at m/z 256.263, 284.293 and 312.325 was based on their abundant fragments at *m/z* 102 ($C_5H_{10}NO$) and *m/z* 116 ($C_6H_{12}NO$). The presence of a single unsaturation in the alkyl chain of acyl amides alters product ion dramatically, as fragmentation differedwith daughter ions corresponding to the combined neutral losses (-35Da) of H_2O and NH_3 in the amide group. Distinct fragments at *m/z*

247 for the successive losses of water and ammonia moieties along with multiple losses of -CH_2 wererecognized in MS^2 spectra of assigned unsaturated acyl amides. Conclusively, the whole loss of the acyl chain and formation of 9-carbon and 10-carbon macrocyclic dieneyl cation yielded daughter ions at *m/z* 135 and 121, and aided in structural elucidation of that subclass(Murphy, 2014).

Peaks 17, 18, 21, 22, 26, 27 and 28 exhibited molecular ions $[M+H]^+$ at*m/z* 256.26, 282.27, 284.29, 310.30, 312.325, 338.34 and 675.67 in MS/MS spectra with distinctive fragment ions of fatty acyl amides;palmitamide, octadecenamide (oleamide), octadecanamide (steramide), eicosenamideicosanamide, erucamide and erucamide dimer, respectively, cluster B in MN (Fig. 4). These metabolites are reported here for the first time in sapota fruit, and likely to account fora wide array of therapeutic indications such as treatment of bacterial infections, cancer, inflammations, and metabolic disorders(Tanvir et al., 2018).Steramide was detected previously in Sapota leaves (Tamsir et al., 2020).

3.3.2. Identification of sphingolipids

The identified sphingolipids were detected in clusters A and C in GNPS network (Fig. 4).Sphingosine is the major form present in this classand is assigned in peaks (3, 6, 8, 10, and 11), followed by the sphinganine class whichwas observed in BPCin peaks (20, 24, 25, and 30).The lipophilicity, formula composition, and fragmentation pattern suggest that these peaks are sphingolipid conjugates.

Most of the sphingolipids and their dihydro equivalents fragment to backbone ions with *m/z* 264 in positive ion mode as a key for the identification of sphingolipids (Otify et al., 2019). Most notably, product ion (*m/z* 284) is for sphinganine, whereas product ion at *m/z* 282 correspondstosphingosine(Suppl. Fig. S2).

For example, peak 8 exhibited a molecular formula [$C_{18}H_{37}NO_3$ (m/z 316.2836)], such formula matches the class of sphingoid bases (that is non-phosphorylated plant sphingolipids) belonging to basic sphingoid compounds, either dehydrophytosphingosine,or 6-hydroxysphingosine(Lénárt et al., 2021). The fragmentation pattern showed product ions at *m/z* 280 and 262 corresponding to losses of 2 and $3H_2O$ molecules, respectively, and assigning it asdehydrophytosphingosine.

Peak 3 showed a fragmentation pattern of tetradecaphytosphingosine, based on the neutral loss of two water molecules and alkyl chain ($C_{10}H_{20}$, 140Da) at *m/z* 226 and 122 (Table 3).Sphingolipid long-chain base(LCB)was detected in peak 5 showingfragment ions at *m\z* 272, 254, 242 (Qu et al., 2018). The cerebroside(peak 29)with $(M+H)^+$at *m\z*732.56 ($C_{40}H_{77}NO_{10}$) and abundant ion at *m\z*570 due to neutral loss of hexosyl and further loss of two water molecules to yield product ion at*m/z*534 (Kang et al., 1999) and assigned as araliacerebroside(Suppl. Fig. S3).

C16 sphinganine, a sphingolipid conjugate, was identified previously in *M. zapota* leaves (Tamsir et al., 2020), albeit this studyrepresents the first comprehensive profiling ofsphingolipidsin sapota fruits.

3.3.3. Identification of Lysophosphatidylethanolamines

Lysophosphatidylethanolamines (Lyso-PE) were characterized in peaks 14and 15 (Suppl. Fig. S4)by the molecular formula of $C_xH_xNO_7P$(Ragheb et al., 2023). LysoPE (0:0/18:2) and LysoPE (0:0/16:0) exhibited$(M+H)^+$at*m/z* 478.29 and 454.29, respectively. The most abundant ions at *m/z* 337 and 313, in their positive-ion mass spectra, corresponded to the neutral loss of 141 Da of phosphoethanolamine(Fang et al., 2003), and aiding in their assignment for the first time in sapota fruit.

3.3.4. Identification of ethanolamines

Peak 9 with $[M+H]^+$ at *m/z* 302 was assigned as tetradecyl diethanolamine ($C_{18}H_{39}NO_2$). The dehydration ofparent ion yielded*m/z* 284, with further cleavage of the carbon chain to yield fragment ion at *m/z* 102. The direct loss of carbon chain from quasi-molecular ion gave product ion at *m/z* 106(Suppl. Fig. S5), key fragmentof this class(Zhang et al., 2022). Peak 13 showed similar fragmentation pattern assigned as N-hexadecyl diethanolamine $[M+H]^+$ at *m/z* 330.33 and fragment ions at *m/z* 312, 106 and 102. This is the first report for the presence ofethanolamines in sapota fruit.Ethanolamines are at the hub of various cellular processes,theystimulate the synthesis of phosphoethanolamine, a vital component to maintain human health. Moreover, ethanolamine prevents cardiovascular disease and ischemia(Patel & Witt, 2017).

3.3.5. Identification of fatty acyl esters

Fatty acyl esters were grouped in cluster D (Fig. 4) (peaks 23&19). This is the first report for the presence of fatty acyl esters in sapota fruit.Peak 23 showed the dehydration of precursor ion$[M+H]^+$ (*m/z* 359) that yielded fragment ion at *m/z* 341(Suppl. Fig. S6) attributed to an allylic cleavage and loss of glyceryl moiety yielding product ion at *m/z* 267 assigned asglyceryl monostearate. Similarly, peak 19 displayed similar fragmentation scheme, suggesting the presence of hexadecanoyl glycerol with product ions at*m/z* 313 and 239.

3.3.6. Identification of Tocopherols

MS^2 fragments of dehydrotocopherol (*m/z* 429.37) were detected in peak 31 and characterized by successive losses of alkyl groups to show fragmentation pattern; (*m/z* 401, 345 and 303), eventually the complete loss of side-chain together with the cleavage of chromene ring developed the product ion *m/z* 165(Suppl. Fig. S7).

3.4. Total Phenolics (TP) and Total Flavonoida (TF) Contents

The quantitative estimation of total phenolics and flavonoids in sapota fruit flesh extract revealed that it encompasses a moderate amount of phenolics (6.79 ± 0.12 mgGAE/g) and traces of flavonoids below our LOQ (limit of quantitation). The ripeness of the fruit results ina major change in itscomposition from an astringent taste owing to tannins and catechins, to a sweet taste due to the elevated sugar content. Fruit ripening had an impact on phenolic content due to the oxidation of phenolic compounds by the action of polyphenol oxidase (PPO) enzyme (Torres-Rodríguez et al., 2011). The higher phenolic and flavonoid contents were reported for leaves, then peels and the least was for the flesh, where values detected were at 14.15 ± 0.48, 1.23 ± 0.06, and0.73 ± 0.1 µg GAE/100 g, respectively, for 70% ethanolextract of each organ(Tamsir et al., 2020).

Comment [WU20]: A table or figure should be added for phenolic and flavonoid contents

3.5. *In vitro* Antioxidant Assays

Assessment ofthe antioxidant activity of sapota fruit pulp extract was carried out using DPPH and ABTS scavenging assays, in addition to FRAP assay to estimate its reducing property. Results revealed moderate effects at 1.62 ± 0.2, 1.49 ± 0.11, and 3.58 ± 0.14 mg TE/g as per DPPH, ABTS, and FRAP assays, respectively. According to previous a reports, the highest antioxidant activity was exhibited by leaf (92.96 ± 0.06 %), then peel (91.98 ± 0.71%), much higher than that of fruit pulp (78.21 ± 0.04 % of DPPH scavenging activity) as was reported in this study(Tamsir et al., 2020).

Comment [WU21]: Make sure DPPH in trolox as per previous comment.

The metabolic profilingof fruit pulp showed that sphingoid bases and fatty acyl amides were the most abundant components, they could be one of the major contributors to the antioxidant activities.Prior studies proved that sphinganine inhibits the transport of cholesterol and low-density lipoprotein(Tamsir et al., 2020),(Roff et al., 1991). Furthermore, previous findings confirm that monounsaturated fatty acids regulate several biochemical events within the cells (Murphy, 2015). Additionally, other constituents may work together synergistically to boost antioxidant effectiveness. Herein, several metabolites detected in the present studywere reported for their antioxidant effect, including sugar alcohols, *viz*, mannitol(Kang et al., 2007), allyl isothiocyanates (Caglayan et al., 2019), palmitic acid, linoleic acid, (Henry et al., 2002), organic acids, *viz*, malic acid (Gąsecka et al., 2018), along withanethole (Aprotosoaie et al., 2019), curlone(Jayaprakasha et al., 2002), limonene (El Omari et al., 2023), cineole (Hoch et al., 2023).

Comment [WU22]: Name the process or events

Comment [WU23]: Add a reference to support synergic effect

3.6. *In Vitro* Enzymes Inhibition Assays

Fruit pulp extract was assessed for its hypolipidemic and antidiabetic activities *viain vitro* assaystargeting the inhibition of pancreatic lipase (PL) and α-glucosidase enzymes, respectively.

PL inhibitory assay tested the extract's influence on enzymatic activity and its potential for obesity management and lipid metabolic disorders. Results revealed that sapota fruit extract inhibited lipase enzyme by IC_{50}= 4.42± 0.5 and 2.21 ± 0.25 at sample concentrations of 10 and 5 mg/mL, respectively,compared to the standard drug, Orlistat which showed IC_{50} valuesof 0.16 and 0.08mg/mL, Table (4).

Comment [WU24]: Units?

Comment [WU25]: Units?

Comment [WU26]: Is it µg or mg

Lipase enzyme plays a major role in fat metabolism. its downregulation leads to a decrease in low density lipoprotein (LDL) and an increase in high density lipoprotein (HDL) (Liu et al., 2020), and provides health benefits for obesity prevention, management and its related disorders (Marzouk et al., 2024).

In the current study, the major metabolites detected in sapota fruit were sphingolipids, fatty acyl amides and phospholipids which could relate to its potential lipase inhibitory effect.Previous studies reported the potency of dietary sphingolipids in improving metabolic syndrome andassociated disorders including atherosclerosis and obesity(Wang et al., 2021). Additionally, the supplementation of sphingolipids has been found to decrease plasma triglycerides, and low-density lipo-protein-cholesterol levels and enhance glucose clearance(Snel et al., 2010). Fatty acyl amideare involved in metabolic heamostasis of human system(Tanvir et al., 2018).

Likewise, phospholipids, amphiphilic lipids rich in sapota pulp,have been implicated in exhibiting a favored impact on blood lipids by reducing TG, total cholesterol, and LDL levels(Küllenberg et al., 2012).Moreover, terpenoids detected by GC/MS were well-reported as pancreatic lipase inhibitor (Singh et al., 2015).Compared to the potential lipase inhibition effect in fruit pulp, no effect was observed regards α-glucosidase inhibitory action. Fruits were found inactive against the enzyme compared to the positive drug control acarbose.

Conclusion

Sapota fruit, *Manilkara zapota* L.*,* iswell recognized for its delightful taste and satisfying flavor, though with less evidence on its comprehensive chemical makeup. In this study, a metabolites profiling approach for sapota fruit pulp was investigatedtargeting its non volatile and volatile chemicals using UPLC/MS and GC/MS techniques. SPME-GC/MS analysis resulted in the detection of 17 aroma compounds belonging to nitrogenous isothiocyanates, ethers, terpenes, aldehydes, acids, alcohols, furan and ketone, which emphasized the fruit's delightful, fragrant aroma. With regards to nutrient metabolites to mediate for fruit value and sensory attributes,GC/MS analysis revealed 68 peaks belonging to sugars (mainly fructose, glucose, mannose, and sucrose), sugar acids, and sugar alcohols, as major components and to account for fruit sweetness, and high-calorie content, in addition to some fatty acids/ esters, organic, inorganic acids, alcohols, terpenes, and nitrogenous compounds. HR-UPLC/MS/MS visualized,using GNPS molecular networking, 31 metabolites, including sphingolipids, fatty acyl amides,lysophosphatidylethanolamines,diethanolamines andfatty acyl esters, which were annotated for the first time in sapota fruit.For standardization of fruit pulp in terms of its total phenolics and flavonoids, moderate level of phenolics was detected at 6.79 ± 0.12mgGAE/g.

The antioxidant assays revealed a moderate free radical scavenging effect via DPPH (1.62 ± 0.2mg TE/g) and ABTS (1.49 ± 0.11mg TE/g) assays, and moderate reducing capacity by FRAP assay (3.58 ± 0.14 mg TE/g). Fruit pulp methanol extract exerted a considerable pancreatic lipase inhibitory (PL) action, compared to the standard drug, Orlistat, which has yet to be clarified for which exact chemical, alongside identifying best solvent to be used to insure best recovery of bioactives targeting such an effect. On the other hand, fruits showed no *a*-glucosidase inhibition

effect likely attributed for moderate levels of phenolics and the absence of flavonoids in their pulp.

The current article presented a comprehensive profiling of phytochemicals to providebetter insight into sapota fruitsnutritiveand health benefits. Future research is recommended to get the best routes for fruit consumption and processing. A comparative approach for exploring metabolites from peels, seeds or fruits from different originswill enhance our insight into the nutritional richness of the fruit and reveal comprehensive chemical profiling. Also, the promising antioxidant and lipase inhibitory action of fruit pulp motivates investigating various extracts to determine the most effective onefor discovering promising natural antiobesity agents~~medications.~~

Comment [WU27]: Conclusion is very large. It may be reduced to 40%

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