QUANTIFICATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS ISOLATED FROM PAWPAW FRUIT (*ASIMINA TRILOBA*)

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by

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and hereby certify that, in their opinion, it is worthy of acceptance.

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ABSTRACT

Pawpaw (*Asimina triloba*) is a North American Annonaceae tree. Pawpaw is an important Native American food and medicine. Inflammation, wounds, gastrointestinal issues, and respiratory disorders have been treated with various parts of the plant. Despite its use, limited research on *A. triloba's* chemical composition and biological activity related to its traditional use is available.

In chapter two, we conducted metabolomic studies based on untargeted analysis to identify possible bioactive compounds found in pawpaw fruit. Ultra-High Performance Liquid Chromatography (UHPLC) system coupled to a maXis impact quadrupole-time-of-flight high-resolution mass spectrometer (Q-TOF) (Bruker Co., Billerica, MA, United States) was used to analyze the pawpaw extracts. The raw data collected from the UPLC-HRMS was analyzed by utilizing the global metabolomic XCMS database. The MS information generated from UPLC-HRMS was uploaded to XCMS platform and analyzed using pairwise job mode to identify the relative abundance of the compound in different cultivars.

In chapter three, the compounds identified through untargeted analysis were quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The LC-MS/MS analyses were performed using an HPLC system (Water Alliance 2695, Water Co., Milford, MA, United States) coupled with a Waters Acquity TQ triple quadrupole mass spectrometer operated in negative and positive electrospray ionization modes.

In chapter four, the putatively identified compounds were assessed for their total antioxidant capacity using the CUPRAC assay. The influence of the compounds on the Nrf2/ARE pathway was determined through the Antioxidant Response Element (ARE)

activation assay. The cytotoxicity of the compounds was evaluated against leukemia (HL-60), melanoma (SKMEL5), breast cancer (MCF-7), ovarian cancer (SKOV3), and prostate cancer (PC3) cell lines. The antibacterial activity of the compounds was tested against *Pseudomonas aeruginosa, Staphylococcus aureus, Mycobacterium smegmatis*, and *Escherichia coli*.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The North American pawpaw (*Asimina triloba* [L.] Dunal) is a fruit tree that belongs to the same family (Annonaceae) as several widely cultivated tropical fruit trees such as soursop/graviola (Annona muricata L.), custard apple (Annona reticulata L.) and sugar apple (Annona squamosa L.) (Brannan et al., 2015a).

The pawpaw stands out as one of the limited temperate species within its family and is indigenous to the Eastern region of North America. It thrives in areas characterized by hot summers and cold winters. Its habitat stretches from western New York to southwestern Ontario, extending southwards through Michigan, Illinois, Missouri, and reaching as far south as eastern Texas and Florida (**Figure 1.1**). Currently, the fruit is cultivated in various other countries, such as South Korea, Japan, Italy, China, Israel, Romania, Portugal, Nigeria, and Belgium (Duffrin & Pomper, 2006; Nam, Jang, et al., 2018). There are approximately 60 commercially available pawpaw cultivars, known for their excellent fruit quality, which are propagated clonally through grafting or budding (Kentucky State University, 2021). This process involves taking scion wood from superior trees and grafting it onto common seedling rootstock, which comes from various genetic sources. Past research has identified more than 40 bioactive acetogenins and other compounds in the crude extracts of different parts of the North American pawpaw, including twigs, unripe fruits, seeds, root, and bark (Ratnayake et al., 1992; G. Zhao et al., 1992). The bioactivity of these acetogenins in pawpaw twigs varies with the seasons,

peaking during the growing season in May and June. During this time, the concentrations of asimicin, bullatacin, and trilobacin, which are the active compounds, increase significantly (Ratnayake et al., 1992).

Pawpaw fruits display mostly asymmetrical features, often taking an oblong-cylindrical shape, although some may have globular or arched forms (Szilagyi et al., 2016). Across different cultivars, there are variations in growth and ripening rates, as well as physical attributes such as size, color, texture, and the percentage of seeds present(Duffrin & Pomper, 2006). While the general taste of the fruit is often likened to a combination of banana, mango, and pineapple flavors, trained sensory panelists have used other descriptors such as apple, melon, and fresh flavors to characterize specific cultivars of pawpaw fruit (Pomper et al., 2010).

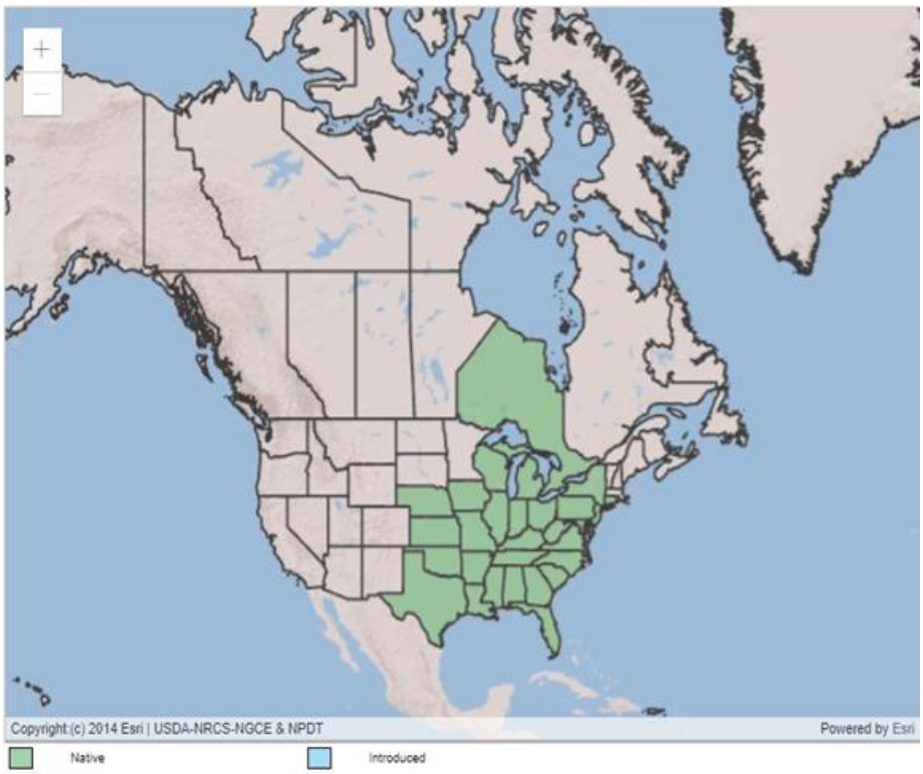

Figure 1. 1. Pawpaw distribution within the United States (USDA)

1.2 Pawpaw nutrients and health-promoting benefits

1.2.1 Nutrition information

When comparing pawpaw with banana, apple, and orange, pawpaw has higher protein and fat content. However, bananas surpass pawpaw in terms of food energy and carbohydrate content. There is minimal difference among these fruits in dietary fiber content. Overall, pawpaw is most similar to bananas in their nutritional composition. Apples are notably low in protein, while oranges are low in fat, and both fruits have lower food energy compared to pawpaw and bananas. **Table 1.1**. summarizes the nutritional comparison of pawpaw with other fruits (National Research Council, 1989).

Pawpaw contains three times the amount of vitamin C compared to apples, twice the amount compared to bananas, and one third the amount compared to oranges. When it comes to riboflavin, pawpaw has six times the quantity found in apples and twice that of oranges. In terms of niacin content, pawpaw surpasses bananas by double, apples by fourteen times, and oranges by four times (**Table 1.1**).

Pawpaw and banana are rich sources of potassium, containing approximately double the amount found in oranges and triple the amount found in apples. In terms of calcium content, pawpaw has 1.5 times more than oranges and roughly ten times more than both bananas and apples. When it comes to phosphorus, pawpaw boasts two to seven times the quantity present in bananas, apples, or oranges. Furthermore, pawpaw exhibits four to twenty times more magnesium, twenty to seventy times more iron, five to twenty times more zinc, five to twelve times more copper, and a striking sixteen to one hundred times more manganese compared to the levels found in bananas, apples, or oranges (**Table 1.1**).

The protein in pawpaw contains all of the essential amino acids. Pawpaw exceeds apple in all of the essential amino acids, and it exceeds or equals banana and orange in most of them. The profile of fatty acids in pawpaw is preferable to that in banana. Pawpaw has 32% saturated, 40% monounsaturated, and 28% polyunsaturated fatty acids. Bananas has 52% saturated, 15% monounsaturated, and 34% polyunsaturated fatty acids (**Table 1.1**) (National Research Council, 1989).

Table 1.1. Nutritional Comparison of Pawpaw with Other Fruits [a]

	Units	**Pawpaw**	**Banana**	**Apple**	**Orange**
	Composition				
Food Energy	Calories	80	**92**	59	47
Protein	grams	**1.2**	1.03	0.19	0.94
Total Fat	grams	**1.2**	0.48	0.36	0.12
Carbohydrate	grams	18.8	**23.4**	15.25	11.75
Dietary Fiber	grams	2.6	2.4	**2.7**	2.4
	Vitamins				
Vitamin A	RE [b]	8.6	8	5	**21**
Vitamin A	IU [c]	87	81	53	**205**
Vitamin C	milligrams	18.3	9.1	5.7	**53.2**
Thiamin	milligrams	0.01	0.045	0.017	**0.087**
Riboflavin	milligrams	0.09	**0.1**	0.014	0.04
Niacin	milligrams	**1.1**	0.54	0.077	0.282
	Minerals				
Potassium	milligrams	345	**396**	115	181
Calcium	milligrams	**63**	6	7	40

Phosphorus	milligrams	**47**	20	7	14
Magnesium	milligrams	**113**	29	5	10
Iron	milligrams	**7**	0.31	0.18	0.1
Zinc	milligrams	**0.9**	0.16	0.04	0.07
Copper	milligrams	**0.5**	0.104	0.041	0.045
Manganese	milligrams	**2.6**	0.152	0.045	0.025
	Essential amino acids				
Histidine	milligrams	21	**81**	3	18
Isoleucine	milligrams	**70**	33	8	25
Leucine	milligrams	**81**	71	12	23
Lysine	milligrams	**60**	48	12	47
Methionine	milligrams	15	11	2	**20**
Cystine	milligrams	4	**17**	3	10
Phenylalanine	milligrams	**51**	38	5	31
Tyrosine	milligrams	**25**	24	4	16
Threonine	milligrams	**46**	34	7	15
Tryptophan	milligrams	9	**12**	2	9
Valine	milligrams	**58**	47	9	40

[a] Mean value per 100 grams edible portion. Pawpaw analysis was done on pulp with skin, although the skin is not considered edible. Probably much of the dietary fiber, and possibly some of the fat, would be thrown away with the skin. Number in bold face represents the highest value for each component.

[b] Retinol Equivalents – these units are used in the most recent National Research Council Recommended Dietary Allowances table (1989).

[c] International Units – these units are still seen on many labels.

1.2.2 Health promoting benefits

The pawpaw fruit has a taste that resembles a combination of mango, pineapple, and banana, and is rich in nutritive components such as vitamins and minerals

(Templeton et al., 2003). Pawpaw trees generate natural substances called annonaceous acetogenins in their leaves, bark, and twigs. These compounds exhibit potent antitumor and pesticidal characteristics (Grygorieva et al., 2021). Throughout history, American pawpaw has been employed to alleviate fever, vomiting, and inflammation in the mouth and throat. Furthermore, the fruit has laxative properties, while the leaves act as a diuretic and are applied externally to treat boils, ulcers, and abscesses (Callaway, 1992; Grygorieva et al., 2021). As the commercial importance of pawpaw continues to grow, several research studies have delved into different aspects of its physiology. McGrath and Karahadian (1994) conducted a study to detail the physical, chemical, and sensory attributes of pawpaw. Wood and Peterson (1999) focused on examining the lipids present in the fruit. Furthermore, Kobayashi et al. (2008) demonstrated that the phenolic content and antioxidant capacity of pawpaw fruit vary depending on the ripeness stage. These investigations contribute valuable insights into understanding the potential uses and benefits of pawpaw in various industries. The study conducted by Ortutu et al. (2015) provided an evaluation of the antioxidant activity of different tropical fruits, including mango, pawpaw, and guava, at various stages of maturation. However, it's worth noting that previous research has primarily concentrated on edible fruit, leading to a gap in knowledge regarding other plant tissues like roots, twigs, and leaves of the pawpaw tree. Additionally, despite the increasing interest in pawpaw, no studies have been conducted on the phenolic acid compounds present in this fruit, which could hold valuable insights into its potential health benefits and applications. Further exploration in these areas would contribute significantly to our understanding of the full range of beneficial properties that pawpaw trees may offer.

Phytochemicals are widely recognized for their positive effects on human health, and among them, phenolic compounds are especially associated with antioxidant activity (Dillard & German, 2000). Nevertheless, the presence and quantity of phenolic compounds can vary significantly among different plant varieties and tissues, as well as under the influence of growing seasons, agronomic practices, and environmental conditions (Dillard & German, 2000; Song et al., 2016). These variations emphasize the need for thorough investigation and understanding of the factors that influence the composition of phenolic compounds in plants to harness their potential health benefits effectively.

The study conducted by Song et al. (2016) revealed variations in the content of polyphenols among different parts of the plant, including roots, stems, leaves, and flowers. Notably, they found that the antioxidant activity was particularly higher in the roots compared to the other tissues. This observation can be attributed to the root's elevated content of polyphenols, as these compounds are closely associated with antioxidant properties. These findings highlight the importance of understanding the distribution of bioactive compounds within different plant parts, as it can have implications for potential health benefits and applications of the plant.

Acetogenins, potent cancer cell inhibitors, are found in the roots, twigs, and seeds of pawpaw trees (Nam et al., 2017). Additionally, studies have demonstrated that pawpaw leaf essential oil exhibits remarkable activity against various cancer cell lines (Ko et al., 2011a; Nam et al., 2017). Furthermore, pawpaw contains procyanidins that possess antioxidant properties, as stated by Brannan et al. (2015). Annonacin is one of the compounds that has been identified in the bark and seeds of *Asimina triloba*, commonly

known as pawpaw, prairie banana, poor man's banana, Ozark banana, Banango, and sometimes referred to by regional names like 'banana' (e.g., Indiana/Hoosier banana, Kentucky banana, etc.). This tree species is prevalent in the Eastern United States. Notably, due to its potential value, pawpaw has been considered as a possible alternative cash crop to tobacco. The presence of annonacin in specific parts of the pawpaw tree highlights its significance in research and may hold implications for various applications and industries (McLaughlin, 2008a).

1.3 Applications in agroforestry

Pawpaw has significant potential for being included in agroforestry production systems, which involve integrating trees and shrubs with crops or livestock in a designed manner. Being a valuable species native to the eastern U.S., pawpaw can play a pivotal role as the main crop or as a supporting tree species within these systems (**Figure 1.2**). Its presence can bring about a wide range of environmental benefits, thereby enhancing the overall conservation value of the agroforestry system (e.g., alley cropping). When the primary objective is to optimize fruit production, whether it be in a dedicated orchard or as part of an agroforestry system like alley cropping, it is essential to use improved cultivars. These improved varieties should be planted at appropriate spacing, ensuring they receive ample sunlight. Additionally, providing irrigation and protection from wildlife damage is crucial to ensure successful fruit production and overall system productivity (Byers et al., 2022).

Alley cropping is a well-suited agroforestry technique for pawpaw production. This practice involves planting rows of trees, which can consist of either pawpaw alone or a mix of pawpaw with other trees or shrubs, while the alleyways between rows are

utilized for companion crops. To prevent water competition, it is advisable to use irrigation, and one option for the alleyway could be a berry shrub like aronia planted between the trees within the row. A diverse array of crops can be chosen for inclusion in the alleyways, such as vegetables, low-growing fruits, or cut flowers. These crops can serve as a source of income for the farmer before the pawpaw trees reach full productivity. On the other hand, pawpaw can be incorporated as a secondary crop in a poly-culture alley cropping system that is designed to optimize the production of tree nuts, for instance. Many tree nut crops take several years to become productive and require additional years to achieve full production and mature canopy diameter. By planting the nut trees at their final optimal spacing (e.g., 60 feet for pecan, 40 feet for Chinese chestnut), it leaves enough room for one to two pawpaw trees to be planted between each nut tree. The pawpaw will become productive earlier than the nut trees, but at some point, the shade from the overstory nut trees would result in a decline in the pawpaw fruit production. Apart from alley cropping, pawpaw can also be effectively integrated into riparian forest buffers, serving to enhance conservation efforts, and contributing to a secondary production function. In such systems, the decision of whether to invest in higher-cost improved cultivars for pawpaw would depend on the specific design and maintenance program in place. Given that pawpaw naturally grows in river floodplains, it becomes an excellent component of a riparian buffer that encompasses a diverse range of tree species. While pawpaw thrives in the understory of larger trees within the buffer, the fruit production may be limited due to the shaded environment. Nevertheless, the colonies formed by pawpaw through root suckers play a crucial role in stabilizing the soil and reducing erosion in the sensitive riparian zone. This makes

pawpaw an asset in the overall functioning and conservation of the riparian ecosystem (Byers et al., 2022).

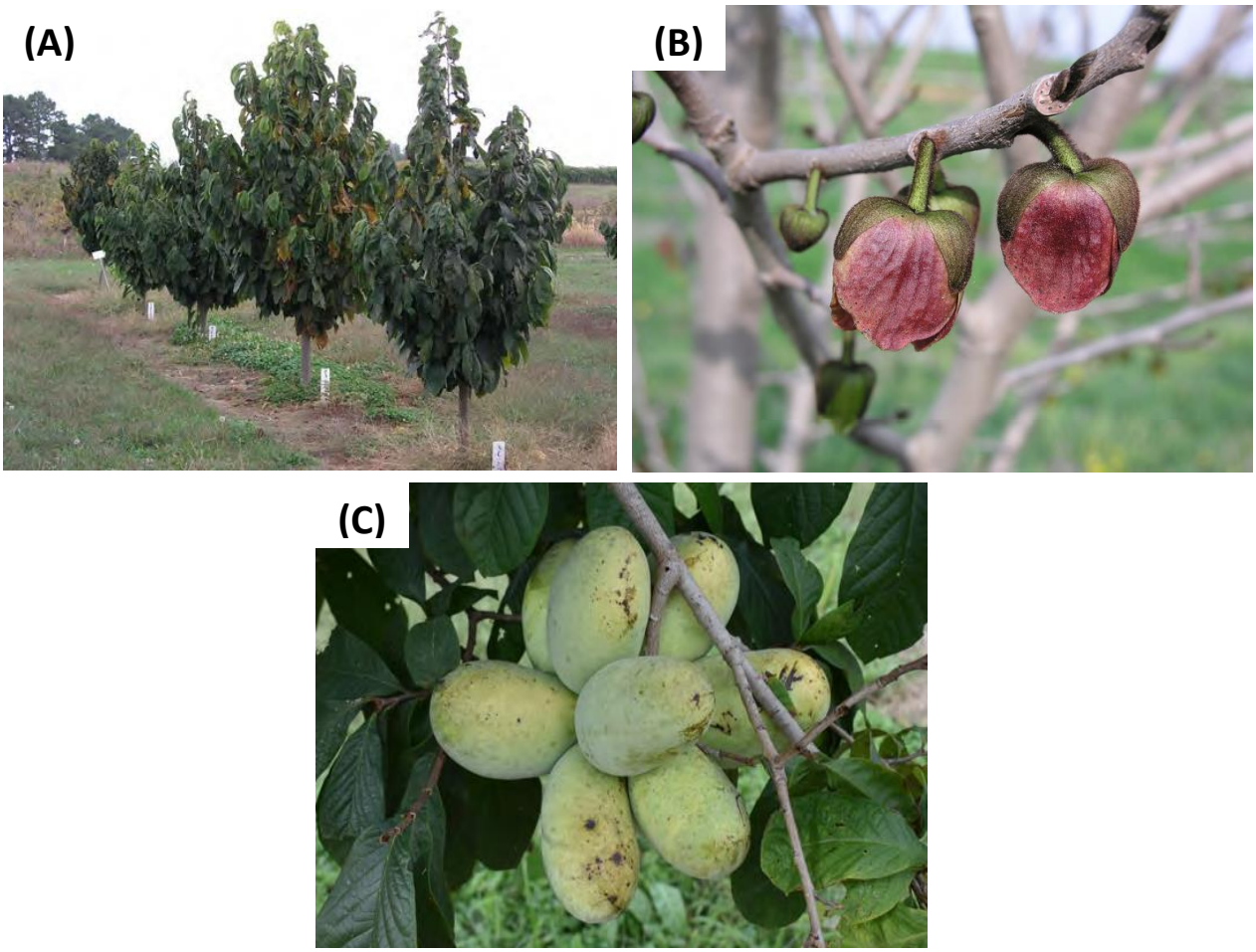

Figure 1. 5. (A) Pawpaw trees, (B) blossoms and (C) fruit (Byers et al., 2022)

1.4 Site Selection

Pawpaw thrives in woodland planting sites with well-drained soils that tend to remain moist, particularly in areas near streams, but they should not be exposed to frequent waterlogging. For establishing pawpaw orchards, it is essential to select well-drained soil areas located close to woodlands or timber plantings. These nearby wooded areas can act as effective windbreaks to protect the orchard from strong winds. While

papaws can grow in shaded environments, optimal fruit production occurs in areas with full sunlight. In native patches where shading is prevalent, fruit set may be low due to factors such as limited pollinators (flies) and insufficient cross-pollination, which requires at least two genetically different pawpaw trees. It is important to avoid planting pawpaw in low-lying valleys or areas with poor air drainage, as these locations can result in the pooling of cold air during spring. This situation can lead to frost damage to pawpaw flowers in April and May, potentially causing crop failure. Careful consideration of the planting site is crucial to ensure successful pawpaw cultivation and fruitful orchard yields (Crabtree & Pomper, 2020).

1.5 Pawpaw Marketing

Pawpaw is a high-value specialty niche cropthat can be sold and consumed fresh. The emerging pawpaw market appears to be profitable and has potential to grow. Since 2016, the Center for Agroforestry, University of Missouri, has conducted surveys to identify pawpaw market trends. In the United States, the supply chain for pawpaw involves several key players, including tree growers, fruit growers, pickers, value-added producers, distributors, retailers, and wholesalers. The majority of pawpaw producers are small-scale and part-time, with their annual gross sales income from pawpaw being less than $5,000. About 15% of producers are engaged in pawpaw businesses as their full-time occupation (Byers et al., 2022; Cai et al., 2019). Despite the potential of the pawpaw market, there are various barriers that have hindered its development. One significant challenge is acquiring the knowledge and skills required to successfully grow, process, and market pawpaw products. Additionally, obtaining high-quality grafted pawpaw trees

can be difficult at times due to an unstable supply. Furthermore, fresh pawpaw fruit has a relatively short shelf life, which can pose challenges for storage and distribution. Addressing these barriers and promoting education and support for pawpaw producers could help unlock the full potential of the pawpaw market in the United States (Byers et al., 2022; Cai et al., 2019).

Recent studies have provided evidence that the market for pawpaws is experiencing growth, as evidenced by several factors. One indicator of this growth is the rising market prices for pawpaws. For instance, the retail price for fresh pawpaws in Rhode Island reached $10.47 per kilogram in 2015, representing a notable increase of 37% compared to the price of $7.23 per kilogram (in 2015 dollars adjusted for inflation) recorded in 2009 (Cai et al., 2019). In addition to increasing market prices, economic profitability is another factor supporting the growth of the pawpaw market. As more consumers express interest in this unique fruit, demand is on the rise, creating opportunities for producers to achieve better economic returns. Overall, the combination of rising market prices, economic profitability, and growing consumer interest in pawpaws indicates a promising trend for the future of the pawpaw industry. These positive developments may encourage further investments and advancements in pawpaw cultivation, processing, and marketing (Pomper et al., 2008). The frozen pawpaw pulp produced by Integration Acres was sold to consumers at a price of $13.23 per kilogram in 2016, indicating a market demand and willingness of consumers to pay a premium for this product (Cai et al., 2019). This price suggests a positive economic outlook for pawpaw producers and indicates a profitable market for frozen pawpaw products. In addition to the direct market price, a cost–benefit analysis conducted in Kentucky further

supports the profitability of pawpaw production. According to the analysis, growers can expect to break even within 6–7 years after planting the pawpaw trees. Moreover, the wholesale price needed to achieve this break-even point was estimated to be less than $3.53 per kilogram (Pomper et al., 2008). These findings indicate that pawpaw cultivation can be financially viable and may yield positive returns for growers within a reasonable timeframe (Pomper et al., 2008). The combination of market prices, cost–benefit analyses, and consumer interest all point to a positive outlook for pawpaw production, suggesting that it can be a profitable venture for growers and participants in the pawpaw supply chain.

CHAPTER 2: IDENTIFICATION OF BIOACTIVE COMPOUNDS USING METABOLOMIC APPROACH

2.1 Introduction

Pawpaw (*Asimina triloba* [L.] Dunal), a tree that is native to the eastern part of the United States, grows as an understory tree or a dense aggregation of shrub in the temperate woodlands or mesic hardwood forests (Pomper et al., 2008). Among the 10 families in the order Magnoliales, pawpaw belongs to the Annonaceae family, often referred to as the tropical custard-apple family. Several fruits within the Annonaceae family are commercially important such as custard apple (Annona reticulata), soursop/guanábana/graviola (Annona muricata), cherimoya (Annona cherimola) and sugar apple (Annona squamosa) (Callaway, 1992). Historically, pawpaw is harvested in the wild, however it is being cultivated as an orchard crop in several states including Alabama, California, Missouri, Maryland, Michigan, North Carolina, Kentucky, West Virginia, and Ohio (Archbold et al., 2003). The pawpaw fruit is the largest tree fruit native to North America. It is oblong-cylindrical in shape, 3–15 cm long, 3–10 cm wide, and weighs up to 1000 g. The harvest season of pawpaw fruits is mid-August to late September. As pawpaw ripens, soluble solids concentration increases, and the production of volatile flavor compounds is enhanced (Archbold et al., 2003).

Pawpaw is a very nutritious fruit containing significant amounts of vitamin A and C, and high levels of health promoting phenolics such as riboflavin and niacin. Pawpaw trees produce natural compounds (annonaceous acetogenins) in leaf, bark, and twig tissues that possess both highly antitumor and pesticidal properties (Kobayashi et al.,

2008). Traditionally, American pawpaw has been used for treating fever, vomiting, and pain and swelling (inflammation) of the mouth and throat. Determining the levels of bioactive compounds in pawpaw may benefit the commercialization of the fruit (Kobayashi et al., 2008). **Figure 2.1** shows the chemical structure of major compounds found in pawpaw. Asimilobin has been isolated from an ethanolic extract of pawpaw's seed and showed potential anti-cancer activity (Woo, Cho, et al., 1995). Coreximine, isoquinoline alkaloids, has shown strong anti-bacterial activity (McLaughlin, 2008b). Considering anti-diabetic and anti-inflammatory potential, epigallocatechin has shown both activities (Baluchnejadmojarad & Roghani, 2011). Finally, uvaricin has shown anti-tumor activity (L. McLaughlin et al., 1986)

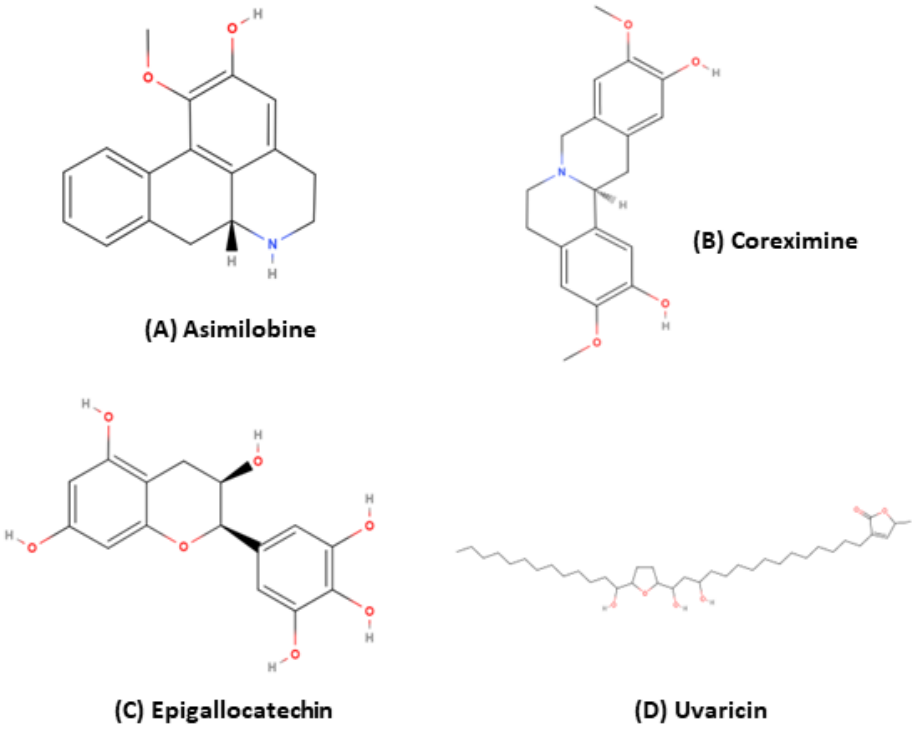

Figure 2. 1. Chemical structure of major compounds found in pawpaw. (A) Asimilobin; (B) Coreximine; (C) Epigallocatechin; (D) Uvaricin.

As a result of recent advancements in mass spectrometry, metabolomics algorithm, computational capacity, and mass spectral reference databases, untargeted metabolomics has been widely applied to identify bioactive molecules in the complex and organic-rich matrices. Metabolomics is considered one of the omics platforms that aims to study the small molecules (<1500 Da) from biological samples (Gorrochategui et al., 2016). Due to the ability to provide information of *in vivo* metabolic profiles at several stages in the discovery-and-development process, this field of study has become very popular in recent years among other omics (Nicholson et al., 2002). Metabolomics analysis has been broadly employed in many disciplines such as drug discovery (Wang et al., 2012), assessing responses to environmental stress (Shulaev et al., 2008), 2008), cancer detection and development (Kim et al., 2009; Sreekumar et al., 2009), natural product discovery (Rochfort, 2005) and therapeutic effects of drugs for diabetes (Wang et al., 2013). Based on the different categories of data generated, metabolomics data analysis strategies are classified into two different categories: targeted (W. Lu et al., 2008) and untargeted analysis (De Vos et al., 2007). Targeted analysis is used to identify a set of known metabolites where analytical standards are required for the analysis. However, untargeted analysis is used to gather information on as many metabolites as possible in each sample analyzed.

Mass spectrometry (MS) is considered a powerful technology that used as a tool in metabolomics with the ability to analyze low molecular weight compounds. High resolution mass- spectrometers (HR-MS) are the most powerful analyzers currently available with an improved of mass determination accuracy (Gorrochategui et al., 2016). The high-resolution mass spectrometry (HRMS) is a mass analyzer which has a mass

resolving power (Rp determined at fullwidth half-height maximum, FWHM) higher than 10,000 (Rathahao-Paris et al., 2016). Low-resolution mass spectrometers, such as quadrupoles and linear ion traps (IT), have been substituted by the HR-MS, such as time-of-flight (TOF) (Wilson et al., 2005), quadrupole time-of-flight (Q-TOF) (Weaver et al., 2007), Fourier transform ion cyclotron resonance (FT-ICR) (Brown et al., 2003), and orbit-traps HRMS (Koulman et al., 2009). The HR-MS is able to resolve isomeric (molecules with the same molecular formula but with different chemical structure) and isobaric (molecules with the same nominal mass but differing exact masses) species (Gorrochategui et al., 2016; Rathahao-Paris et al., 2016).

Most recent approach in metabolomics analysis using HR-MS coupled to UPLC has many advantages compared to traditional approach. The ability UPLC-HRMS to accurately measure low molecular weight of metabolites within a few minutes definitely outperforms traditional approaches that typically needs longer time and steps to run (Zhang et al., 2012). Modern metabolomics approaches also provide less labor intensity than traditional approaches. The latest development in modern metabolomic tools is the introduction of ultra-high-performance liquid chromatography (UPLC) that employs porous particles with internal diameters smaller than 2 μm. The UPLCs perform better in small molecular detection especially if coupled with HRMS. The UPLC-HRMS has higher peak capacity, improved sensitivity, and resolution compared to conventional HPLC systems (Zhang et al., 2012).

In this study, ultra-performance liquid chromatography coupled with time-of-flight high resolution mass spectrometry (UPLC-TOF HRMS) has been utilized for identification and triple quadrupole mass spectrometry (LC Triple quad MS/MS) for

quantification of bioactive compounds in pawpaw. Time of flight mass spectrometry (ToF-MS) analyzer can provide accurate mass measurement and a full scan spectral sensitivity due to the excellent ion separation and detection (Lacorte & Fernandez-Alba, 2006). It can record an accurate full scan spectrum throughout the acquisition range. In general, a ToF-MS instrument measures the time an ion needs to travel through a field-free region, as shown in the schematic overview of ToF-MS. The ions generated in the ion source are accelerated as discrete packages into the field-free flight tube by using a pulsed electrical field (Lacorte & Fernandez-Alba, 2006). The triple quadrupole tandam mass spectrometer (TQD) is equipped for targeted analysis due to its ability to offer a better sensitivity, reproducibility, and a broad dynamic range (W. Lu et al., 2008). It can be utilized in multiple reaction monitoring mode (MRM), with a pre-optimized process to get an optimized number of the collision energy and product ion *m/z* ratio that generates the best signal for each analyte (W. Lu et al., 2008).

Untargeted metabolomics approach allows a comprehensive metabolomic profiling in biological samples. In this chapter, we conducted metabolomic studies based on untargeted analysis to identify possible bioactive compounds found in pawpaw fruit. Untargeted metabolomics is the global profiling of small molecules in a system without any bias. Although several analytical techniques can be employed to perform untargeted metabolomics, liquid chromatography coupled with high-resolution mass spectrometry (UPLC-HRMS) has been frequently used because of the large number of molecules that can be evaluated in a single analysis (Tautenhahn et al., 2012). For example, ten to thousands of features (a feature is defined as an ion with a distinctive m/z and retention time) can be detected by high resolution UPLC-HRMS in one extract. In general, the

main purpose of untargeted metabolomics is to determine which of these features is dysregulated (upregulated and downregulated) between different sample groups or treatments. Due to the complexity and the number of features in a dataset, it is challenging to accomplish this comparison manually (Domingo-Almenara et al., 2018). Several software programs for automated processing of UPLC-HRMS data have been developed over the past decade. However, most of these programs have restrictions that limit their utility and applicability to different instrumentation. One widely applicable program for processing LC/HRMS data is XCMS Online, a web-based platform that contains all of the tools necessary for the entire untargeted metabolomic workflow, including signal detection, peak alignment, retention time correction calculations, raw data processing, statistical analysis, and metabolite assignment (Gowda et al., 2014; J. Lu et al., 2019; Vu et al., 2020). **Figure 2.2** represents XCMS online workflow for metabolomic data processing. An untargeted metabolomic profiling approach that utilizes a comprehensive program like XCMS Online is well-suited to the identification of candidate compounds found in pawpaw. XCMS online is coupled with METLIN library, which is a powerful web-based database for metabolites. METLIN originated as a database to characterize known metabolites and has since expanded into a technology platform for the identification of known and unknown metabolites and other compounds. Through this effort it has become a comprehensive resource containing over 1 million molecules including lipids, amino acids, carbohydrates, toxins, small peptides, and natural products, among other classes (Guijas et al., 2018).

In this study, the raw data collected from the UPLC-HRMS was analyzed by utilizing the global metabolomic XCMS database. The MS information generated from

UPLC-HRMS was uploaded to XCMS platform and analyzed using pairwise job mode to identify the relative abundance of the compound in different cultivars.

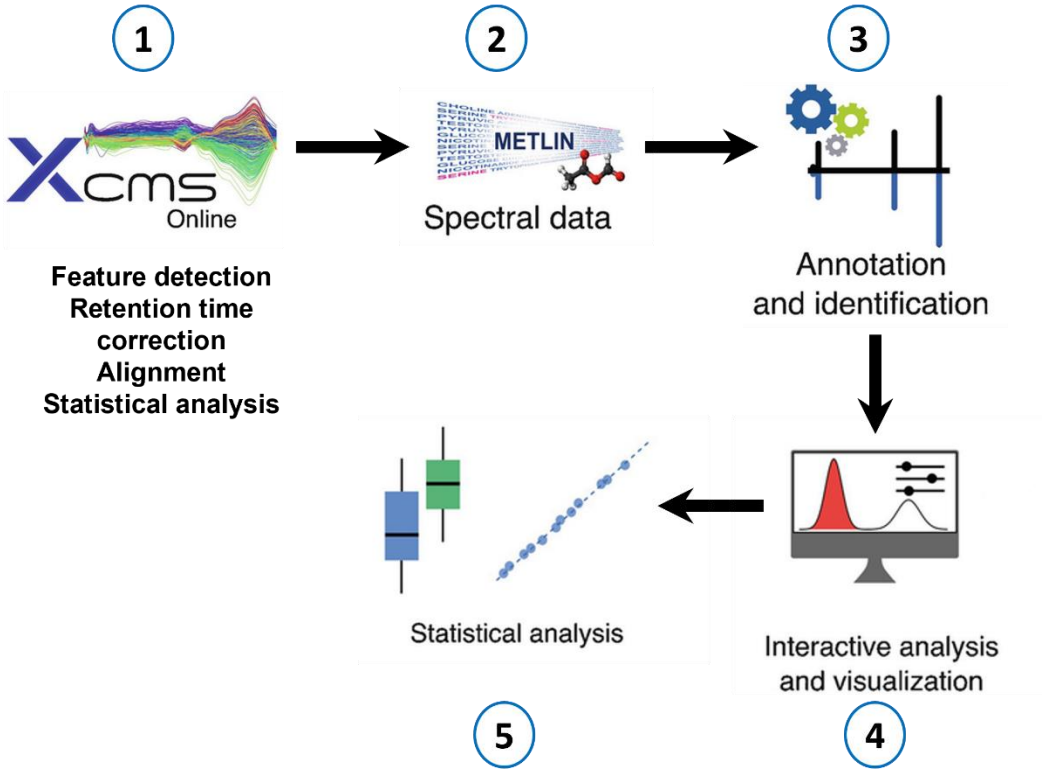

Figure 2. 2. XCMS online workflow for metabolomic data processing.

2.2 Objectives

The objectives of this study are:

1- To identify the bioactive compounds found in pawpaw using metabolomics approach.
2- To quantify relative abundance of the compounds in different cultivars.

2.3 Materials and methods

2.3.1 Sample preparation

All the samples were harvested from the University of Missouri, Southwest Research, Extension, and Education Center (SWREEC) (**Table 2.1**). Approximately 15g (5g dry weight equivalent) of ripe fruit from each cultivar was blended and added to 50 mL methanol (MeOH). The mixture was then sonicated for 60 minutes using sonicator (FS-60, Fischer Scientific, PA, USA) and centrifuged for 30 minutes at 3,000 rpm. The supernatant was collected and filtered through a 0.2 μm syringe filter (Whatman® Anotop®, GE Healthcare, Germany) and transferred to an HPLC vial (Waters, USA) prior to injection into the UPLC-HRMS. There were three replicates for each cultivar. The HPLC-grade methanol was used as blank.

Table 2. 1. Detailed information of the samples used in this study.

Sample ID	Harvest Date	Cultivar	Tree #	Site	Tissue
22	9/9/2020	10-35	9-6	SWC-lower orchard	Ripe fruit
23	9/2/2020	Overleese	5	SWC-upper orchard	Ripe fruit
25	9/2/2020	PA Golden	7-4	SWC-lower orchard	Ripe fruit
27	9/2/2020	Shenandoah	5-4	SWC-lower orchard	Ripe fruit
29	9/2/2020	Sunflower	12	SWC-upper orchard	Ripe fruit
31	9/9/2020	Susquehanna	5-5	SWC-lower orchard	Ripe fruit
33	9/16/2020	Wells	4-3	SWC-lower orchard	Ripe fruit
34	9/2/2020	Wilson	4-1	SWC-lower orchard	Ripe fruit

2.3.2 Untargeted metabolomics global chemical profiling analyses

Ultra-High Performance Liquid Chromatography (UHPLC) system coupled to a maXis impact quadrupole-time-of-flight high-resolution mass spectrometer (Q-TOF) (Bruker Co., Billerica, MA, United States) was used to analyze the pawpaw extracts. The system was operated in either negative or positive electrospray ionization modes with the nebulization gas pressure at 43.5 psi, dry gas of 12 L/min, dry temperature of 250˚C and a capillary voltage of 4000 V. The samples from 8 different cultivars were separated using Waters Acquity UHPLC BEH C18 column (2.1 × 150 mm, 1.7 mm particles size) at 60˚ C. The solvent system was 0.1% formic acid (FA) in water (A) and 100% acetonitrile (B). The gradient elution used started with a linear gradient of 95%: 5–30%: 70% (eluents A: B) in 30 min. Subsequently, the separation was followed by a linear wash gradient as follows 70–95% B, 95% B, 95–5% B, and 5% B at 30–33 min, 33– 35 min, 35–36 min, and 37–40 min, respectively. The flow rate was 0.56 mL/min. Mass spectral data were collected automatically using a scan range from 100 to 1,500 m/z and auto calibrated using sodium formate after data acquisition.

2.3.3 XCMS data processing parameters and statistical analysis

The XCMS data were processed using the following parameters: pairwise jobs between each wastewater extract and the control (methanol) were conducted in centWave mode for feature detection (minimum peak width = 5 s, 1 m/z = 10 ppm, and maximum peak width = 20 s), an obiwarp method was selected for retention time correction (profStep = 1), chromatogram alignment was set as minfrac = 0.5, bw = 5, mzwid = 0.015, max = 100, minsamp = 1, and adducts were optimized for UPLC/Bruker Q-TOF in both positive and negative ESI mode . An unpaired parametric Welch t-test was used for

the statistical analysis. Metabolites of significant features ($p < 0.001$ and intensity ≥10,000) were putatively identified by the integration of the METLIN database with XCMS Online.

2.4 Results and discussion

XCMS online provides a complete untargeted metabolomic analysis ranging from the computationally extensive raw data processing, retention time correction calculation, to statistical analysis and metabolite annotation (Tautenhahn et al., 2012). The data files were converted based on XCMS algorithm and presented for peak detection, peak grouping, spectra extraction, and retention time (RT) alignment and correction. The pairwise job offered metabolite comparison based on statistical analysis of two groups (pawpaw extracts and MeOH). XCMS online provides several figures including Total Ion Chromatogram (TIC) and Cloud plot analysis. TIC after RT correction and alignment in positive and negative ion mode presented in **Figure 2.3**.

A cloud plot generated as a default XCMS output (**Figure 2.4**) shows a dysregulated feature in m/z versus RT, represents ions whose intensities are adjusted between samples groups according to statistical thresholds set on p-value < 0.001 and fold change > 1.5. Size of the circles is relative to their average difference in relative intensity of the peak between samples and blank (Tautenhahn et al., 2012). The up-regulated features are presented as green circles on the top of the plot, while the down-regulated features are presented as red circles on the bottom. The shade of color of each features represented the p-value, where darker circles displayed as features with higher p-values (Welch t test, unequal variances) (Tautenhahn et al., 2012).

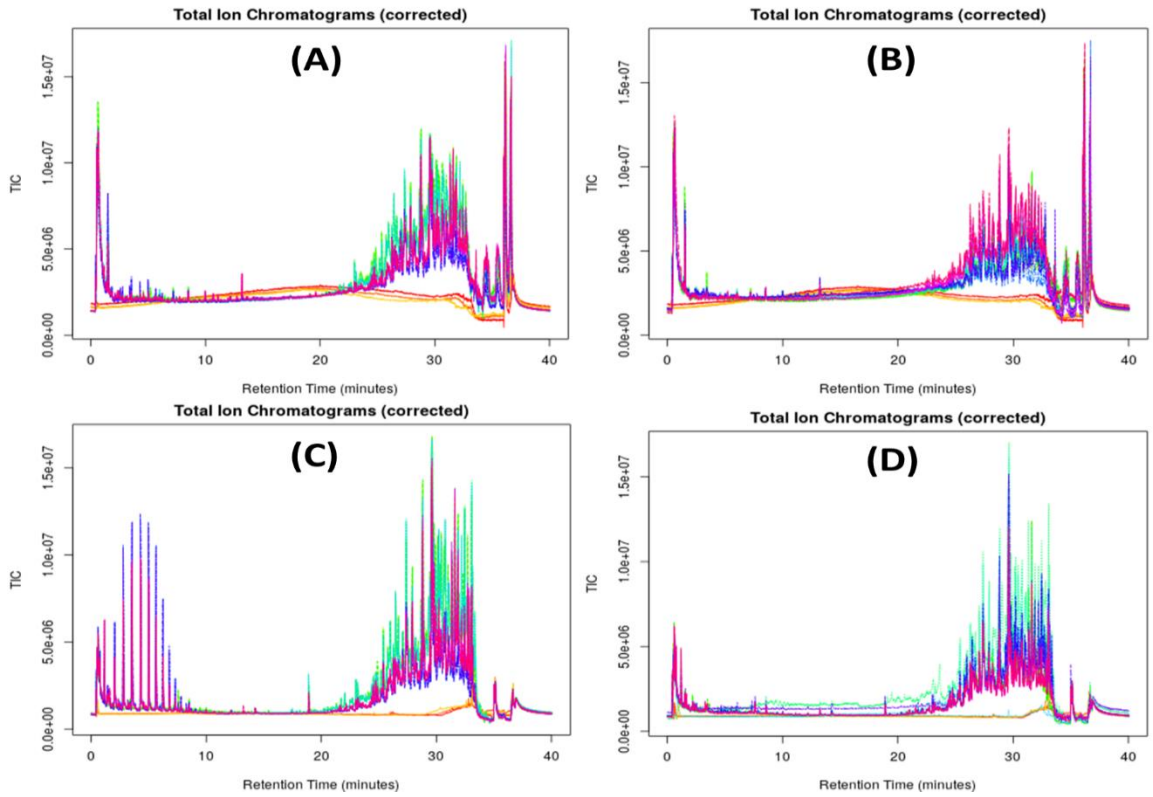

Figure 2. 3. Representative of total ion chromatograms (TIC) of pawpaw. (A) Samples 22, 23, 25, 27 in negative ion mode; (B) Samples 29, 31, 33, 34 in negative ion mode; (C) Samples 22, 23, 25, 27 in positive ion mode; (D) Samples 29, 31, 33, 34 in positive ion mode.

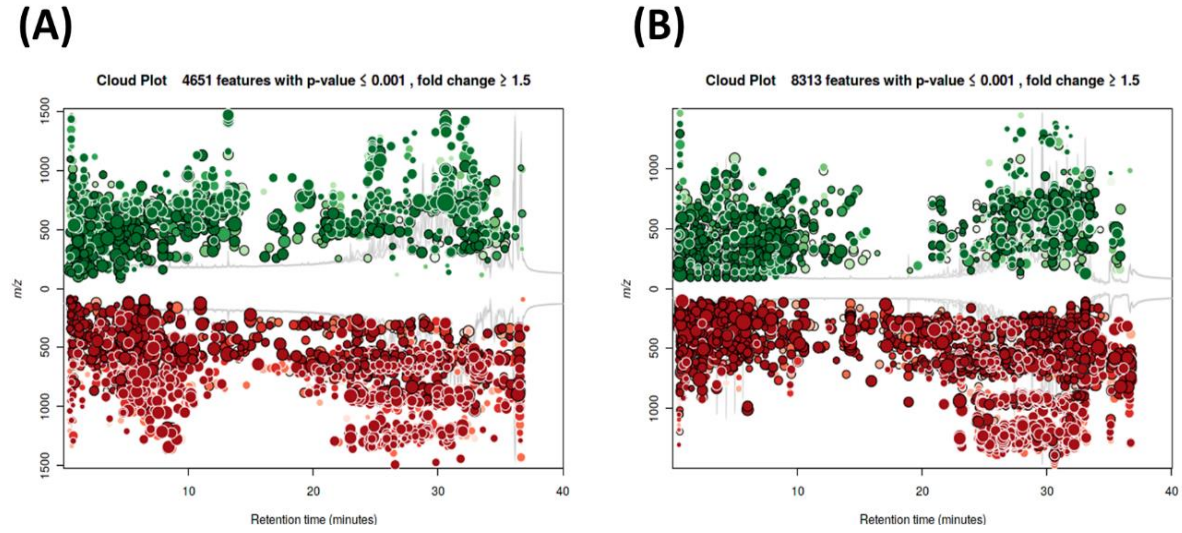

Figure 2. 4. Metabolomic cloud plot. (A) Between samples 22 and 27 in negative ion mode; (B) Between samples 22 and 23 in positive ion mode.

XCMS online also provides a multi-group analysis to compare metabolite features between samples with more than three groups. Principal component analysis (PCA) is one of the multivariate analyses that can be extracted from XCMS online. The results from PCA analysis between multiple groups are presented in **Figure 2.5**. The PCA result from cultivars 22, 23, 25, and 27 in negative ion mode demonstrated that based on the 40% variance at the first principal component and 15% of the second principal component (**Figure 2.5 A**). The PCA results from the same cultivars in positive ion mode were based on 45% variance of PC1 and 19% of PC2 (**Figure 2.5 C**). However, 35% variance at PC1 and 19% PC2 was observed for the cultivars 29, 31, 33, and 34 in the negative ion mode (**Figure 2.5 B**). Finally, The PCA results from the same cultivars in positive ion mode were based on 40% variance of PC1 and 16% of PC2 (**Figure 2.5 D**).

In this study, METLIN database which equipped with over 960,000 metabolites has been scanned for different classes of bioactive compounds. **Table 2.2** shows 54 compounds that were putatively identified in pawpaw extracts.

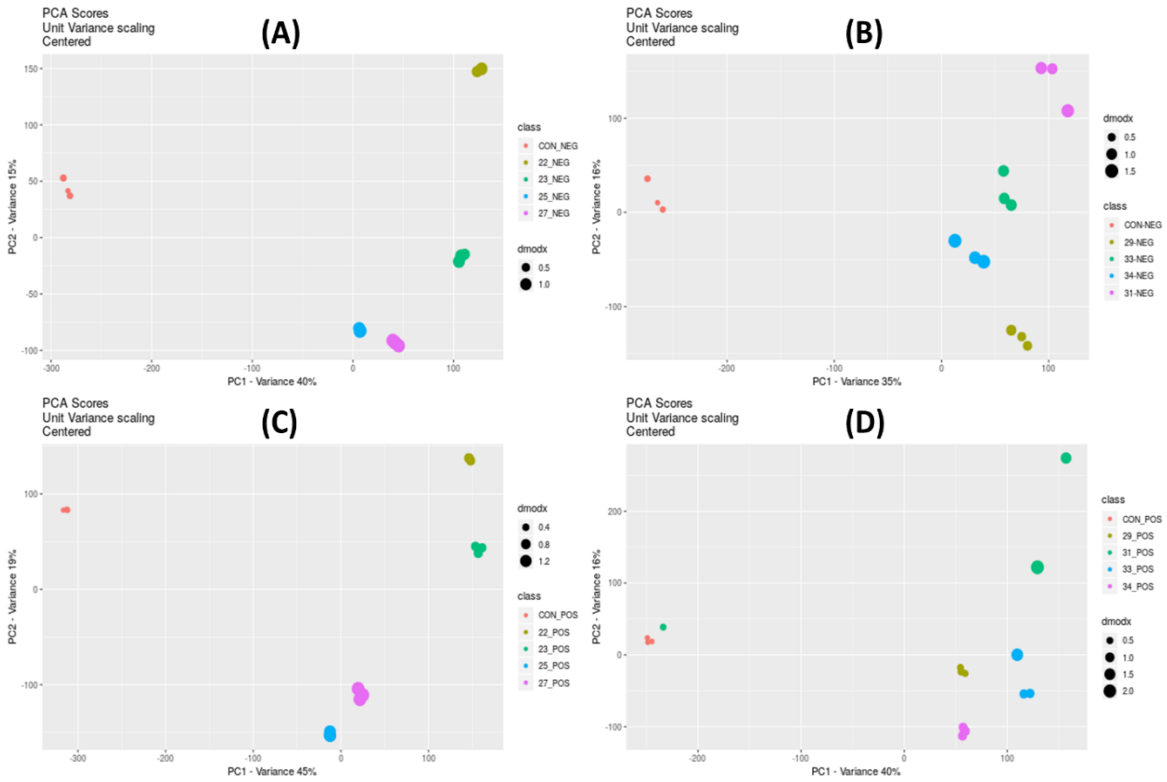

Figure 2. 5. Principal component analysis (PCA) results. (A) Between samples 22, 23, 25, 27 in negative ion mode; (B) Between samples 29, 31, 33, 34 in negative ion mode; (C) Between samples 22, 23, 25, 27 in positive ion mode; (D) Between samples 29, 31, 33, 34 in positive ion mode.

Table 2. 2. List of the compounds putatively identified in pawpaw extracts.

Putatively identified compound	Formula	Retention time (min)	Theoretical mass	Extracted mass	Δppm	Adducts
3,4-Dihydroxybenzoic acid	$C_7H_6O_4$	0.95	172.061	172.061	2.91	$[M+NH_4]$
(-)-Epicatechin	$C_{15}H_{14}O_6$	2.99	290.078	290.086	26.57	[M+H]
(±)-Catechin	$C_{15}H_{14}O_6$	2.99	290.078	291.086	26.57	[M+H]
Chlorogenic Acid	$C_{16}H_{18}O_9$	2.24	354.095	354.088	-21.18	[M-H]
Gallic acid	$C_7H_6O_5$	1.22	170.021	170.029	45.28	[M+H]
p-Coumaric acid	$C_9H_8O_3$	2.55	164.048	164.041	-42.67	[M-H]
Quercetin	$C_{15}H_{10}O_7$	5.11	302.042	302.05	26.15	[M+H]
Rutin	$C_{27}H_{30}O_{16}$	4.98	610.154	610.146	-12.45	[M-H]
ferulic acid	$C_{10}H_{10}O_4$	2.17	194.058	194.051	-37.61	[M-H]
Epigallocatechin	$C_{15}H_{14}O_7$	14.25	324.107	324.106	-6.1	$[M+NH_4]$
Annonacin	$C_{35}H_{64}O_7$	24.74	596.464	596.472	13.41	[M+H]
Uvaricin	$C_{39}H_{68}O_7$	23.18	648.496	648.4554	61.68	[M+H]
Trilobacin	$C_{37}H_{66}O_7$	27.93	622.48	622.488	-12.85	[M+H]
Bullatin	$C_{37}H_{66}O_7$	27.93	622.48	622.488	-12.85	[M+H]
(-)-Epigallocatechin 3-glucuronide	$C_{21}H_{22}O_{13}$	0.78	482.105	482.112	14.5	[M+H]
Muricatacin	$C_{17}H_{32}O_3$	21.05	284.234	284.242	-29.55	[M+H]
Corossolone	$C_{35}H_{62}O_6$	27.64	578.454	578.461	-0.15	[M+H]
Corossoline	$C_{35}H_{64}O_6$	30.58	580.469	580.476	11.12	[M+H]
Lignans	$C_{22}H_{22}O_8$	1.39	414.131	414.095	-15.52	[M+H]
Asiminocin	$C_{37}H_{66}O_7$	30.44	622.48	622.488	13.17	[M+H]
Rolliniastatin-1	$C_{37}H_{66}O_7$	30.44	622.48	622.488	13.17	[M+H]
Asitrocinone	$C_{35}H_{64}O_7$	24.74	596.464	596.472	12.64	[M+H]
N-Feruloyltyramine	$C_{18}H_{19}NO_4$	3.59	313.131	313.139	26.63	[M+H]
(+)-Syringaresinol	$C_{22}H_{26}O_8$	6.47	418.162	419.169	17.8	[M+H]
Squamolone	$C_5H_8N_2O_2$	7.81	146.092	146.094	7.5	[M+H]
Motrilin	$C_{37}H_{66}O_7$	27.93	622.48	622.488	12	[M+H]
Asimilobin	$C_{35}H_{62}O_6$	27.64	578.454	578.461	12.4	[M+H]
cis-Goniothalamicin	$C_{35}H_{64}O_7$	29.62	596.464	596.473	15.1	[M+H]

Trilobalicin	$C_{35}H_{62}O_8$	28.21	610.443	610.452	14.7	[M+H]
Purpureacin-1	$C_{37}H_{66}O_8$	32.18	638.476	638.468	-12.7	[M-H]
Squamocin	$C_{37}H_{66}O_7$	29.35	622.48	622.488	12.6	[M+H]
Parviflorin	$C_{35}H_{62}O_7$	31.6	594.45	594.4424	-12.9	[M-H]
Caffeic Acid	$C_9H_8O_4$	2.13	180.043	180.035	-38.8	[M-H]
Gallocatechin 3-O-gallate	$C_{22}H_{18}O_{11}$	4.73	458.084	458.092	17.4	[M+H]
Naringin	$C_{27}H_{32}O_{14}$	7.2	580.179	580.172	-12	[M-H]
(-)-Catechin gallate	$C_{22}H_{18}O_{10}$	0.65	442.091	442.084	-16.2	[M-H]
Cinnamic acid	$C_9H_8O_2$	1.16	148.053	148.045	-47.2	[M-H]
L-Phenylalanine	$C_9H_{11}NO_2$	1.26	165.079	165.072	-7.87	[M-H]
Quinic acid	$C_7H_{12}O_6$	0.71	192.064	192.056	-35.9	[M-H]
Citric acid	$C_6H_8O_7$	0.64	192.027	192.019	-38	[M-H]
Malic acid	$C_4H_6O_5$	0.61	166.012	166.014	13.8	[M-H]
Ascorbic acid	$C_6H_8O_6$	0.64	176.032	176.025	-41.4	[M-H]
cysteine	$C_3H_7NO_2S$	2.07	121.021	121.013	-62	[M-H]
Tartaric acid	$C_4H_6O_6$	36.16	150.017	150.01	-6.6	[M-H]
Succinic acid	$C_4H_6O_4$	0.77	118.03	118.019	-67.7	[M-H]
Adriamycin	$C_{27}H_{29}NO_{11}$	5	543.175	543.166	-15.1	[M-H]
Trilobine	$C_{35}H_{34}N_2O_5$	5.88	562.246	562.208	-14.1	[M+H]
Coreximine	$C_{19}H_{21}NO_4$	3.51	327.146	327.154	24.1	[M+H]
(+)-Isocorydine	$C_{20}H_{23}NO_4$	4.9	341.162	341.17	23.7	[M+H]
Liriodenine	$C_{17}H_9NO_3$	7.94	275.057	275.065	30.5	[M+H]
Sitosterol	$C_{29}H_{50}O$	23.03	414.385	414.374	-26.5	[M+H]
Asiminenin A	$C_{37}H_{66}O_6$	31.35	606.485	607.492	11.2	[M+H]
Genistein	$C_{15}H_{10}O_5$	9.78	270.052	270.059	25.9	[M+H]
(+)-Norushinsunine N-oxide	$C_{18}H_{17}NO_4$	3.46	311.115	311.123	25.7	[M+H]

2.5 Conclusion

XCMS online platform allows us to easily identify unknown metabolites due to the METLIN Database. METLIN produces a list of potential metabolite identities based on the exact mass. The raw data from HRMS were processed with the XCMS online platform and the features were annotated using the METLIN library, which resulted in the putative identification of 54 bioactive compounds.

CHAPTER 3: QUANTIFICATION OF BIOACTIVE COMPOUNDS USING HIGH PRESSURE LIQUID CHROMATOGRAPH AND TANDEM MASS SPECTROMETRY (LC-MS/MS)

3.1 Introduction

The search for new plant species, especially neglected and underutilized plant species that are a valuable source of biologically active compounds, and creation on its basis the new generation of nutritional supplements, has recently become an urgent branch of modern biological science, and one of the most important scientific directions (Grygorieva et al., 2021). *Asimina triloba* (pawpaw) (**Figure 3.1**) belongs to the family Annonaceae, native to eastern North America and Canada (Layne, 1996). Pawpaw fruits are rich in vitamins and minerals (Pomper & Layne, 2004), and a good source of potassium and several essential amino acids, and they contain significant amounts of riboflavin, niacin, calcium, phosphorus, and zinc (Galli et al., 2007). Furthermore, they have a high polyphenolic content (Brannan et al., 2012; Harris & Brannan, 2009) and antioxidant content(Donno & Turrini, 2020; Kurahashi et al., 2009).

The unripe fruit extract of A. triloba has both functional food and therapeutic benefits, particularly in the treatment of cancer. It is a naturally derived substance that may have lower toxicity compared to traditional chemotherapy drugs (Nam, Park, et al., 2018). Biologically active compounds are not only in fruits, but in different parts of the plant: roots, bark, twigs, leaves, flowers, and seeds (Hui et al., 1989; G. Zhao et al., 1992; G.-X. Zhao et al., 1994). The roots, twigs, flowers, and seeds of A. triloba contain

acetogenins, which are strong inhibitors of cancer cells (Ko et al., 2011a; Ratnayake et al., 1992; Sica et al., 2016; Woo, Zeng, et al., 1995). A. triloba leaf essential oil has strong activity against cancer cell lines (Alali et al., 1999, 1999).

Figure 3. 1. *Asimina triloba* (pawpaw) fruit (Byers et al., 2022).

The objective of targeted metabolomics analysis is to analyze sets of data that are derived from a specific portion of the metabolome. This portion consists of a predetermined group of metabolites that have been chemically identified and biochemically described and are included in a reference database. Tandem mass spectrometry combined with liquid chromatography (LCMS/MS) is a highly effective analytical technique in metabolomics. It allows the quantification of metabolites in biological samples.

Compared to traditional analytical techniques like GC-MS, HPLC-UV, and LC-MS, the Triple Quad MS, a type of triple quadrupole mass spectrometer, provides superior sensitivity, reproducibility, and a wider dynamic range. This is due to the high selectivity of the ions produced by the instrument. The instrument is used in multiple reaction monitoring mode (MRM) for our targeted analysis, employing a pre-optimized

process to determine the best collision energy and product ion m/z ratio for each analyte. This approach is designed to produce the most effective signal for each target compound.

The triple quad MS consists of a series of quadrupoles, each with a distinct function. The first quadrupole is used to select the desired parent ion, while the second quadrupole serves as a collision cell that fragments the parent ion. Finally, the third quadrupole is utilized to isolate the specific product ion (W. Lu et al., 2008).

A triple quadrupole mass spectrometer is capable of screening a particular precursor ion and quantifying it based on the selected product ion, which effectively eliminates most of the matrix interferences in a sample. By improving the specificity, and enhanced signal to noise ratios, the HPLC-MS/MS approach provides higher resolution separation, enhances method sensitivity, and ultimately lowers the limit of detection (LOD) compared to single MS instruments (Gorrochategui et al., 2016). Therefore, we utilized the triple quadrupole MS/MS instrument for quantification of bioactive compounds found in pawpaw fruit.

3.2 Objectives

This study is aimed at quantifying the concentration of the major bioactive compounds identified in pawpaw's fruit. The specific objectives of this study were:

1. Develop the extraction protocols and LC-MS/MS methods.

2. Estimate the sensitivity of the analytical methods.

3. Quantify the major bioactive compounds found in pawpaw's fruit.

3.3 Materials and methods

3.3.1 Sample preparation

All the samples were harvested from the University of Missouri, Southwest Research Center. Approximately 15g (5g dry weight equivalent) of ripe fruit from each cultivar was blended and added to 50 mL methanol (MeOH). The mixture was then sonicated for 60 minutes using sonicator (FS-60, Fischer Scientific, PA, USA) and centrifuged for 30 minutes at 3,000 rpm. The supernatant was collected and filtered through a 0.2 μm syringe filter (Whatman® Anotop®, GE Healthcare, Germany) and transferred to an HPLC vial (Waters, USA) prior to injection into the HPLC-MS/MS. There were three replicates for each cultivar. The HPLC-grade methanol was used as blank.

3.3.2 Targeted analyses for confirmation and quantification

The compounds identified through untargeted analysis were quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The LC-MS/MS analyses were performed using an HPLC system (Water Alliance 2695, Water Co., Milford, MA, United States) coupled with a Waters Acquity TQ triple quadrupole mass spectrometer operated in negative and positive electrospray ionization modes with the nebulization gas pressure at 43.5 psi, dry gas of 12 L/min, dry temperature of 250℃ and a capillary voltage of 1500 V. Compounds in the pawpaw extracts (30 μL volume per injection) were separated using a Phenomenex Kinetex C18 reverse-phase column (100 × 4.6 mm; 2.6 mm particle size, Torrance, CA, United States) at 40℃. The mobile phases were 0.1% formic acid and 10 mM ammonium acetate in water (A) and 100% acetonitrile (B). The elution gradient used was 2% B (0-0.5 min), 2–80% B (0.5–7 min), 80–98% B

(7.0–9.0 min), 2% B (9.0–15.0 min) at a flow rate of 0.5 mL/min. MS detection was performed by MS/MS using the multiple reaction monitoring (MRM) mode. Waters IntelliStart optimization software was used to optimize collision and ionization energy, MRM and SIR (single ion recording) transition ions (molecular and product ions), capillary and cone voltage, and desolvation gas flow. Waters Empower 3 software was used to analyze data. Concentrations of the compounds found in pawpaw extracts were determined based on a calibration curve for each analyte generated using standards of these compounds (purity > 95%, Sigma-Aldrich) at 8 concentrations (0.01, 0.05, 0.1, 0.5, 1.25, 2.5, 5, 10 mg/L) in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated to assess the sensitivity of the analytical method. For each compound, the signal-to-noise ratios of three and ten were employed to calculate LOD and LOQ, respectively.

3.3.3 Statistical analysis

The averages and the standard deviations of data were calculated using Microsoft Excel 2016. Statistical analyses were conducted using XLSTAT software (XLSTAT, 2018: Data Analysis and Statistical Solution for Microsoft Excel. Addinsoft, Paris, France). Paired samples comparison of variances was used to establish the comparison of the mean. A statistically significant difference was defined as $p < 0.05$.

3.3 Results and discussion

The results of optimized source condition and MRM transition (cone voltage and collision energy), retention times, molecular ions, and characteristic fragment ions were summarized in **Table 3.1.** Optimization process was conducted to determine the optimize

Cone voltage (CV) and Collision energy (CE) that generate better peak signal for the compounds(Bayati et al., 2022; W. Lu et al., 2008). **Table 3.2** summarizes the linear correlation coefficients, detection, and quantification limits for the methods (LOD, LOQ).

Validation of the analytical method can be assessed by calculating various analytical parameters, such as the limit of detection (LOD) and the limit of quantification (LOQ). The LOD and LOQ parameters are utilized to characterize the minimum concentration of a substance being measured that can be reliably detected using an analytical procedure. Although LOD and LOQ are interconnected, they have distinct definitions. LOD refers to the lowest concentration of a substance that can be detected, but not necessarily accurately quantified, without providing information about the precision of the result. On the other hand, LOQ is used to establish the concentration at which quantification becomes feasible, considering bias and precision objectives (Gupta, Vipin, 2011).

Table 3. 1. Optimized MRM/SIR parameters for the compounds in positive and negative ion mode

Compound	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)	Ionization mode	Cone voltage (V)	Collision energy (eV)
Annonacin	595	197.3	12.72	ES-	Tune	Tune
(±)-Catechin	288.87	109.1	6.02	ES-	70	28
3,4-Dihydroxybenzoic acid	152.73	109.1	5.65	ES-	65	16
Caffeic Acid	178.8	135.1	6.37	ES-	55	14
Cinnamic acid	146.95	103.1	8.40	ES-	30	12
Citric acid	190.8	111.1	2.10	ES-	50	12
Epicatechin-(-)	288.98	109.1	6.23	ES-	70	30
Epigallocatechin	304.82	125.1	5.79	ES-	60	20
Ferulic acid	192.8	134.1	7.28	ES-	70	16
Gallocatechin 3-O-gallate	456.8	169.1	6.39	ES-	70	18
Genistein	268.81	133.2	8.52	ES-	35	30
Naringin	578.97	151.1	6.87	ES-	55	46
p-Coumaric acid	162.79	119.1	6.93	ES-	25	14
Quercetin	300.82	151.1	7.98	ES-	50	24
Rutin	608.9	271.1	6.50	ES-	70	64
Trilobatin	434.9	273.2	7.34	ES-	30	16
Isocorydine (+)	342.02	280.02	6.37	ES+	25	20
N-Feruloyltyramine	314.01	177.1	7.69	ES+	10	24

Table 3. 2. Linear correlation coefficients, detection, and quantification limits for the method (LOD, LOQ).

Compound	Linear Equation*	R^2	LOD** (µg/L)	LOQ*** (µg/L)
(±)-Catechin	y = 107.62x - 3894.1	0.98	90.8	302.5
3,4-Dihydroxybenzoic acid	y = 307.44x - 15334	0.98	194	646.7
Caffeic Acid	y = 1218.9x - 6979.9	0.99	16.9	56.6
Citric acid	y = 31.704x - 2387.2	0.99	7.1	23.6
Epicatechin-(-)	y = 29.582x - 536.76	0.99	119.5	398.4
Epigallocatechin	y = 2.6114x - 155.57	0.98	556.4	1854.6
Ferulic acid	y = 124.1x + 2505.5	0.99	149.1	497
Gallocatechin 3-O-gallate	y = 304.17x - 14216	0.93	4.7	15.5
Genistein	y = 1217.9x + 21014	0.99	1.3	4.3
Isocorydine (+)	y = 4047.2x + 68469	0.99	2.3	7.7
Naringin	y = 3075.1x + 12258	0.99	3	9.9
N-Feruloyltyramine	y = 4352.6x + 47891	0.99	15.9	52.9
p-Coumaric acid	y = 5060.3x + 7074	0.99	3.4	11.3
Quercetin	y = 5057.3x - 18029	1	20.7	68.9
Rutin	y = 921.66x - 3862.2	0.99	4.5	14.9
Trilobatin	y = 3772x + 40246	0.99	5.2	17.4

***Linear equations represent the relationship between compound peak area and concentration (µg/L) in standard solutions**
****LOD = Limit of Detection**
*****LOQ = Limit of Quantitation**

Optimized Multiple Reaction Monitoring (MRM) or Single Ion Recording (SIR) parameters are crucial for sensitive and specific quantification of targeted analytes in mass spectrometry-based proteomics or metabolomics experiments. These parameters include precursor ions, product ions, collision energies, dwell times, and other instrument settings. **Figures (3.2-3.10)** represent the optimized cone voltage (CV) and collision energy (CE) for 18 targeted compounds. Molecular ion and product ion selection from optimization process were also reported and represented in Figures **3.11-3.19**.

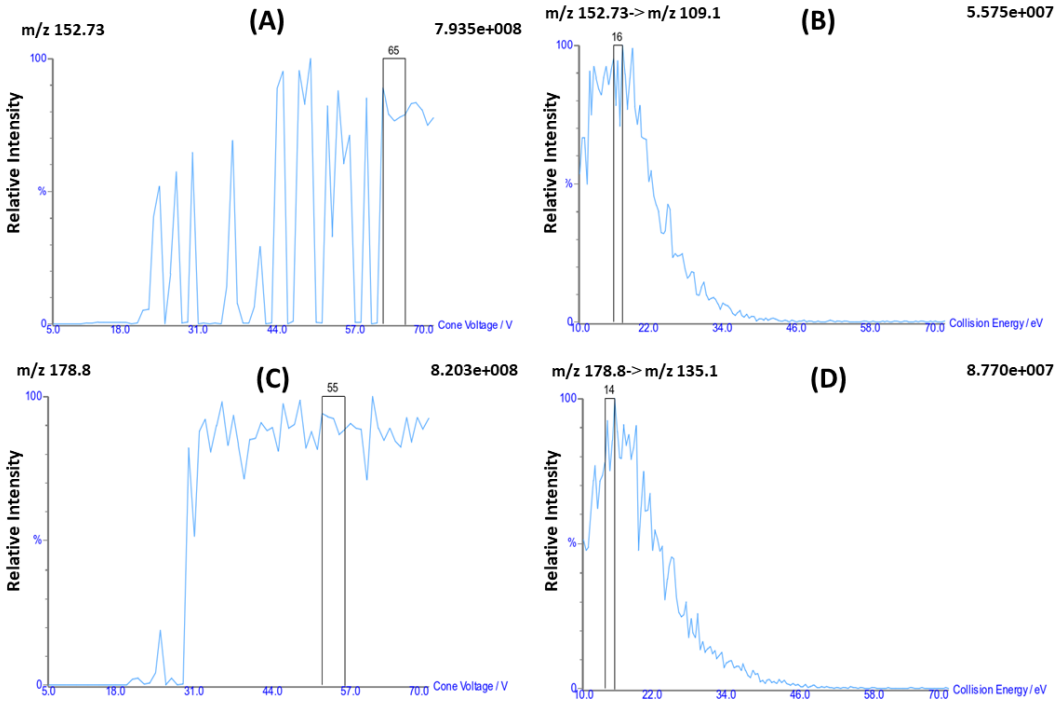

Figure 3. 2. Optimized cone voltage (CV) and collision energy (CE) for (A, B) 3,4-Dihydroxybenzoic acid; (C, D) Caffeic Acid.

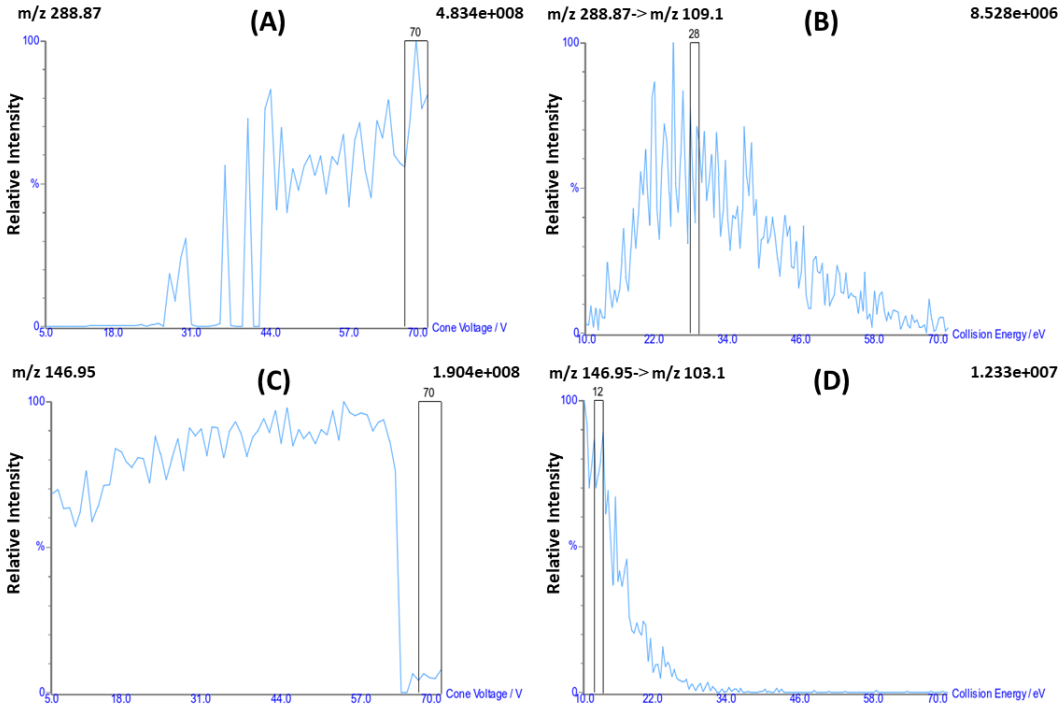

Figure 3. 3. Optimized cone voltage (CV) and collision energy (CE) for (±)-Catechin; (C, D) Cinnamic acid.

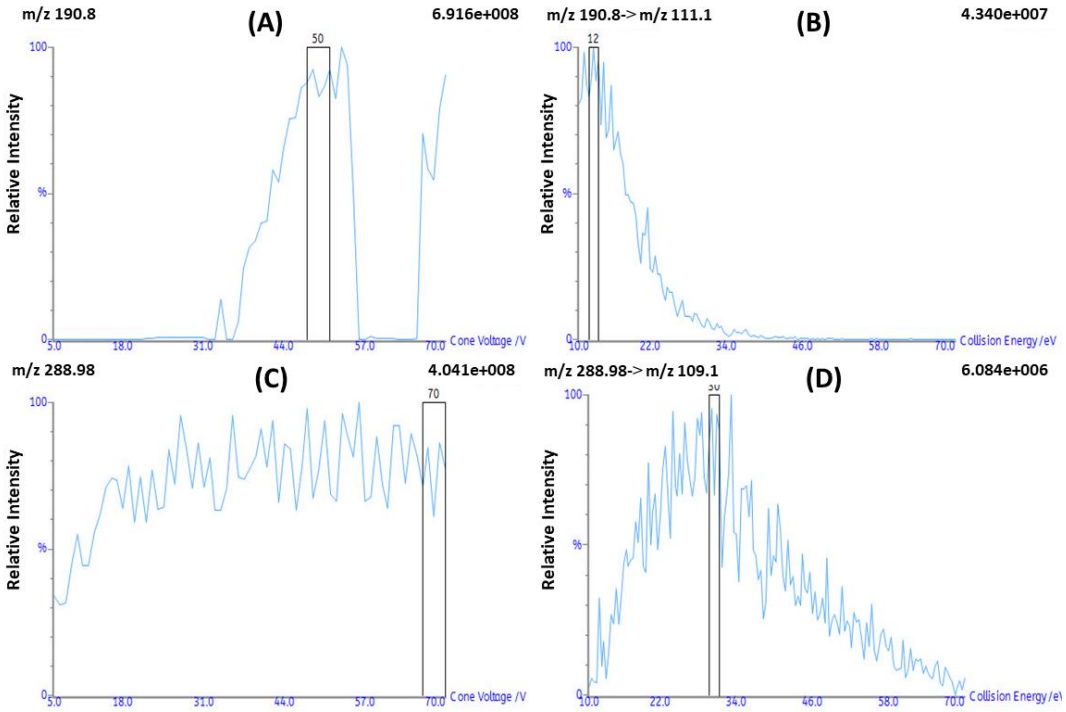

Figure 3. 4. Optimized cone voltage (CV) and collision energy (CE) for (A, B) Citric acid; (C, D) Epicatechin.

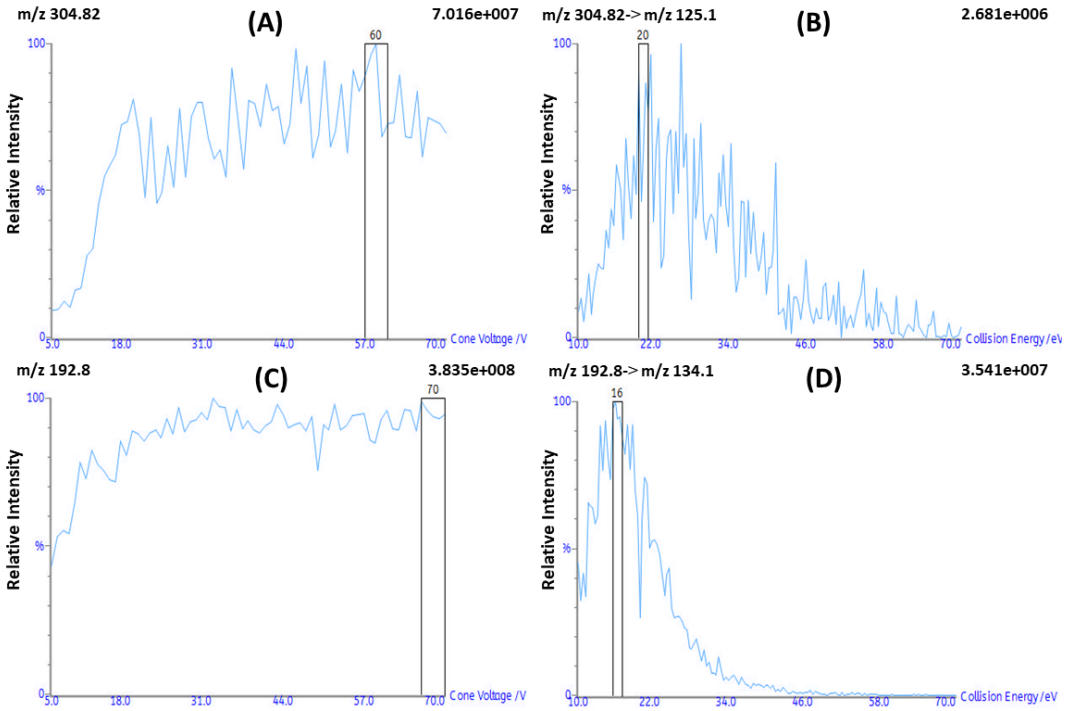

Figure 3. 5. Optimized cone voltage (CV) and collision energy (CE) for (A, B) Annonacin; (C, D) Ferulic acid.

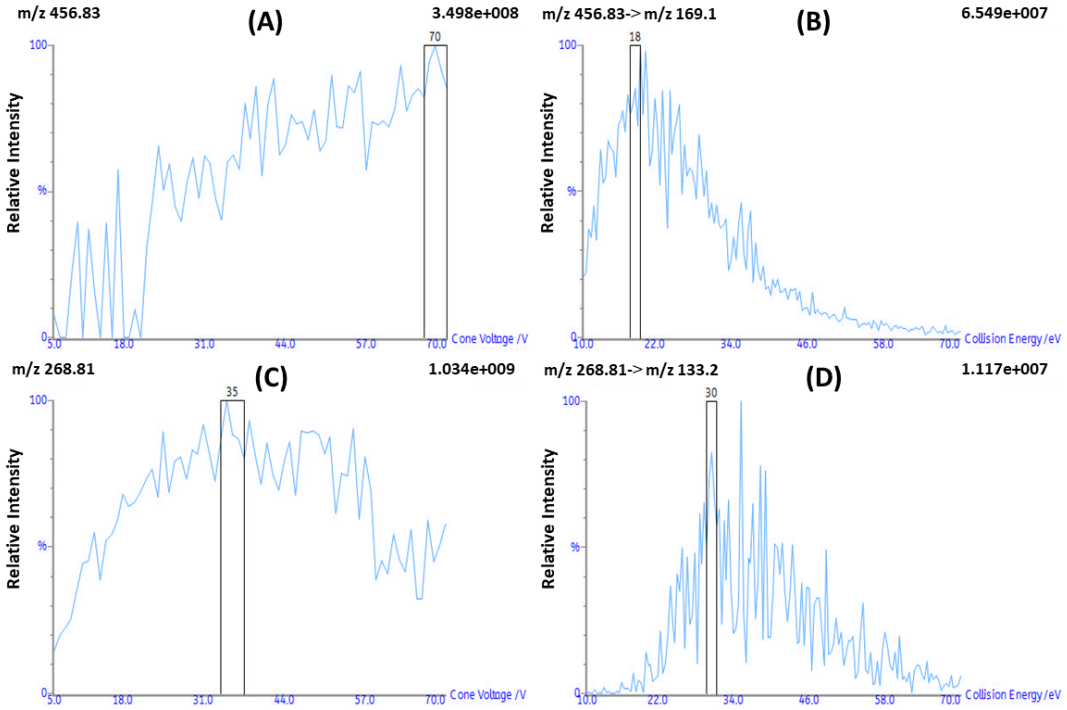

Figure 3. 6. Optimized cone voltage (CV) and collision energy (CE) for (A, B) Gallocatechin 3-O-gallate; (C, D) Genistein.

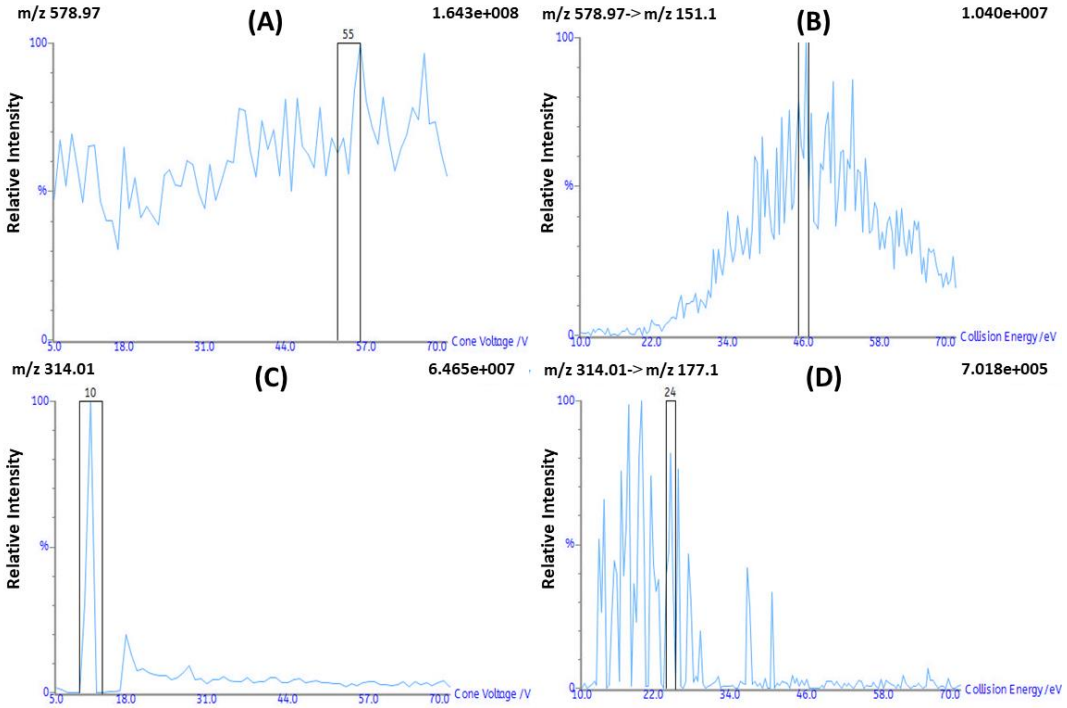

Figure 3. 7. Optimized cone voltage (CV) and collision energy (CE) for (A, B) Naringin; (C, D) N-Feruloyltyramine.

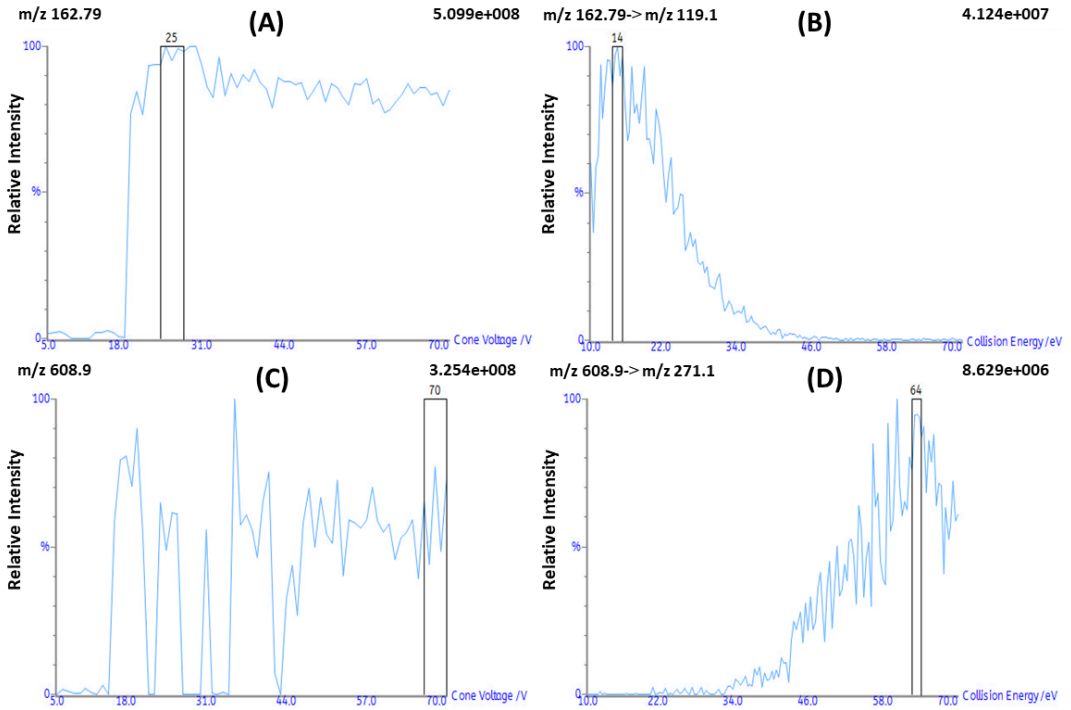

Figure 3. 8. Optimized cone voltage (CV) and collision energy (CE) for (A, B) p-Coumaric acid; (C, D) Rutin.

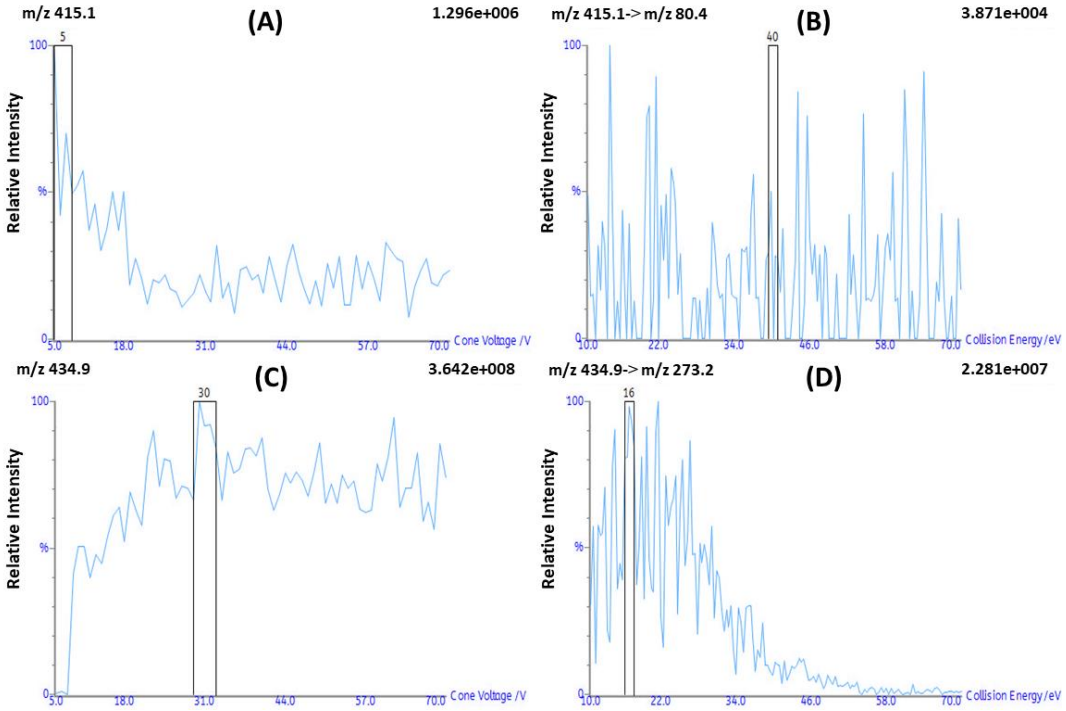

Figure 3. 9. Optimized cone voltage (CV) and collision energy (CE) for (A, B) Sitosterol; (C, D) Trilobatin.

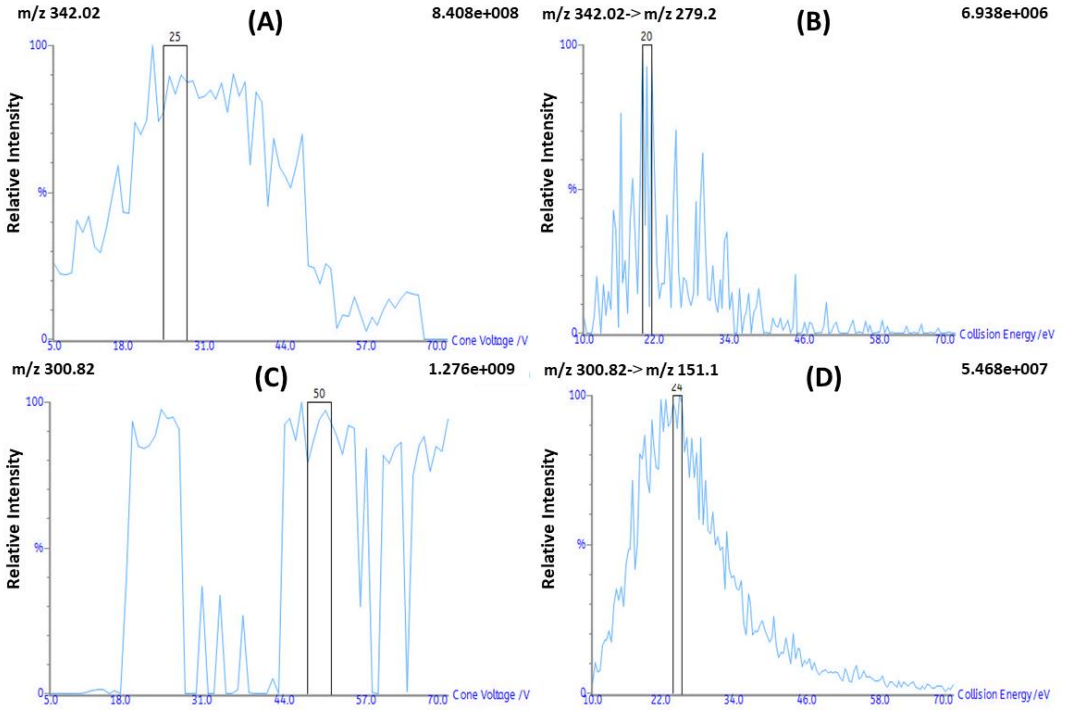

Figure 3. 10. Optimized cone voltage (CV) and collision energy (CE) for (A, B) Isocorydine; (C, D) Quercetin.

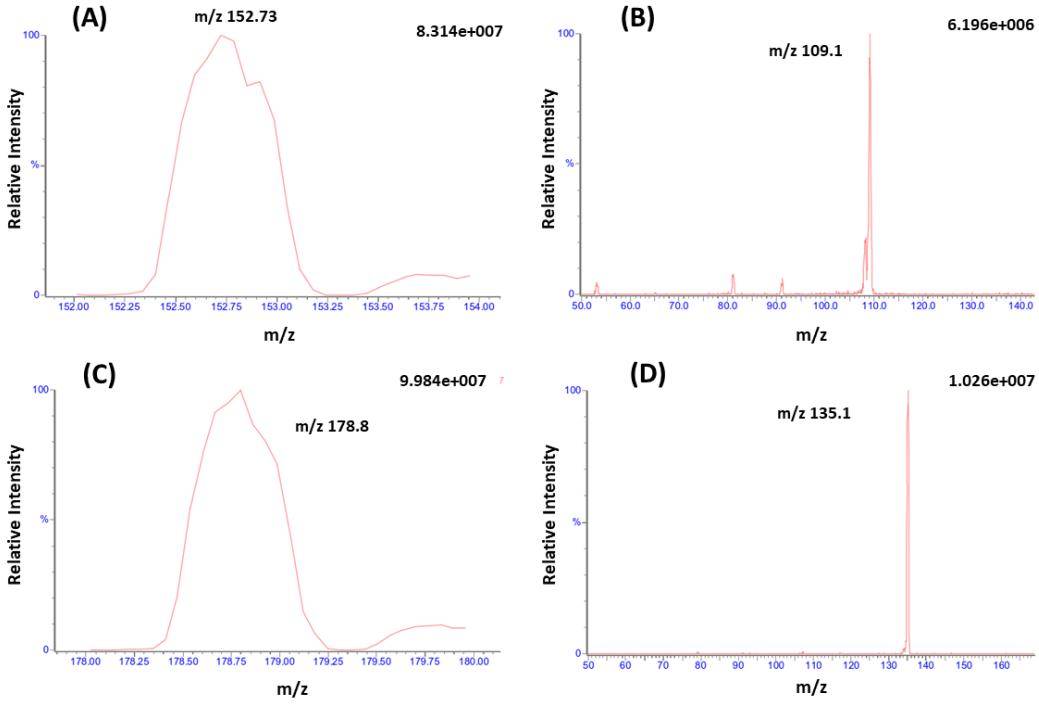

Figure 3. 11. Molecular ion and product ion selection from optimization process for (A, B) 3,4-Dihydroxybenzoic acid; (C, D) Caffeic Acid.

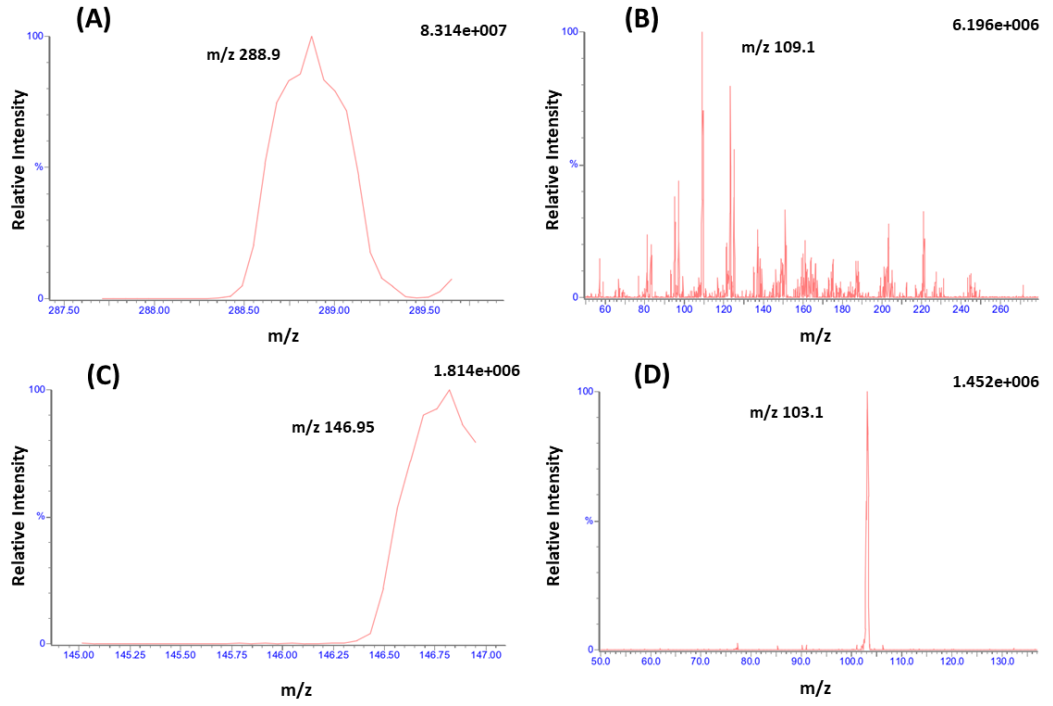

Figure 3. 12. Molecular ion and product ion selection from optimization process for (A, B) (±)-Catechin; (C, D) Cinnamic acid.

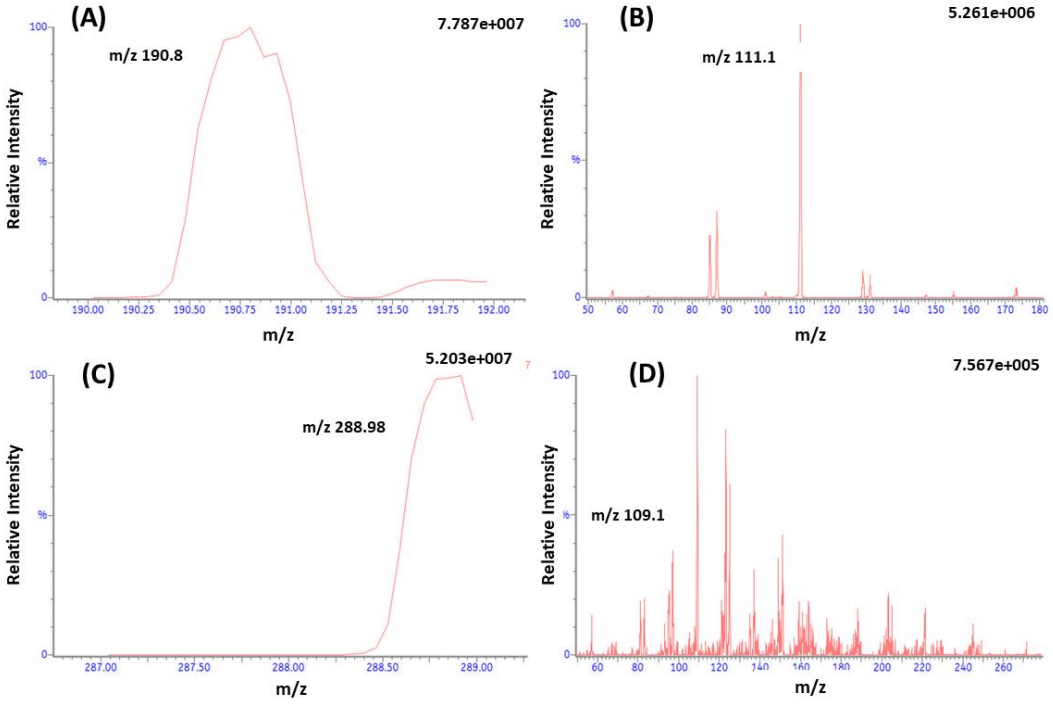

Figure 3. 13. Molecular ion and product ion selection from optimization process for (A, B) Citric acid; (C, D) Epicatechin.

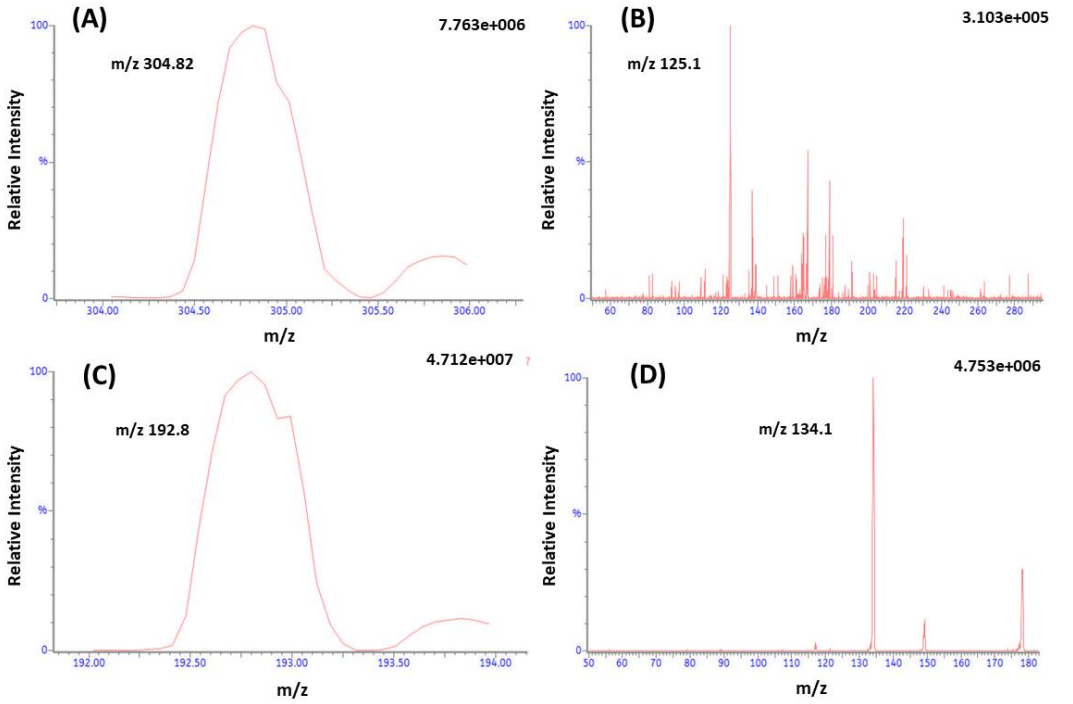

Figure 3. 14. Molecular ion and product ion selection from optimization process for (A, B) Annonacin; (C, D) Ferulic acid.

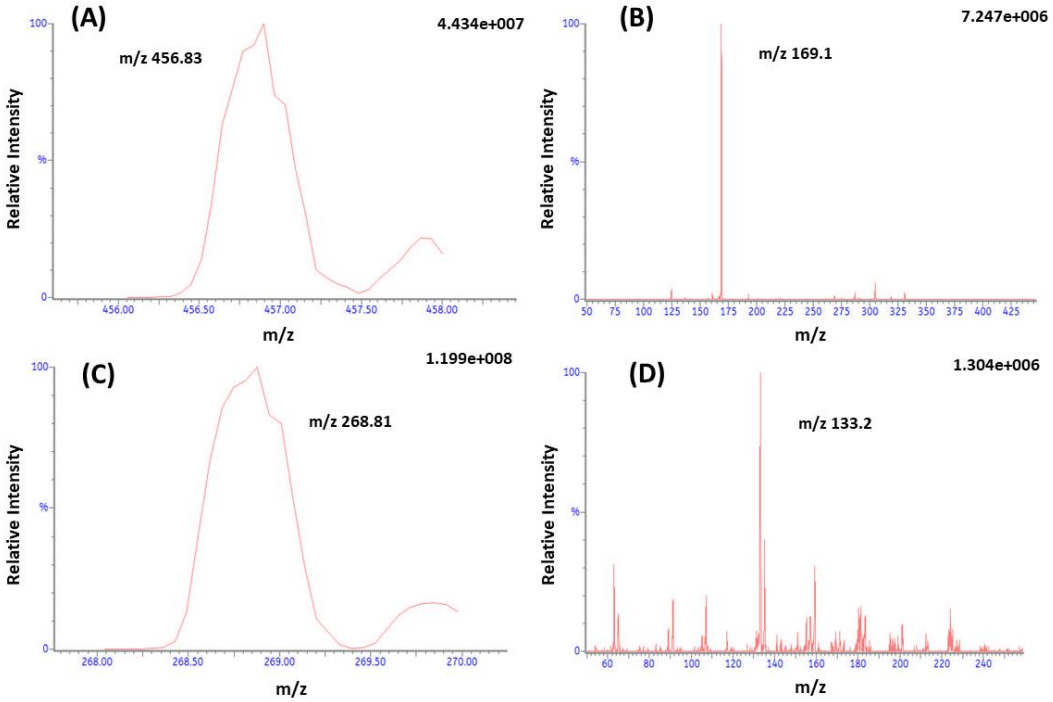

Figure 3. 15. Molecular ion and product ion selection from optimization process for for (A, B) Gallocatechin 3-O-gallate; (C, D) Genistein.

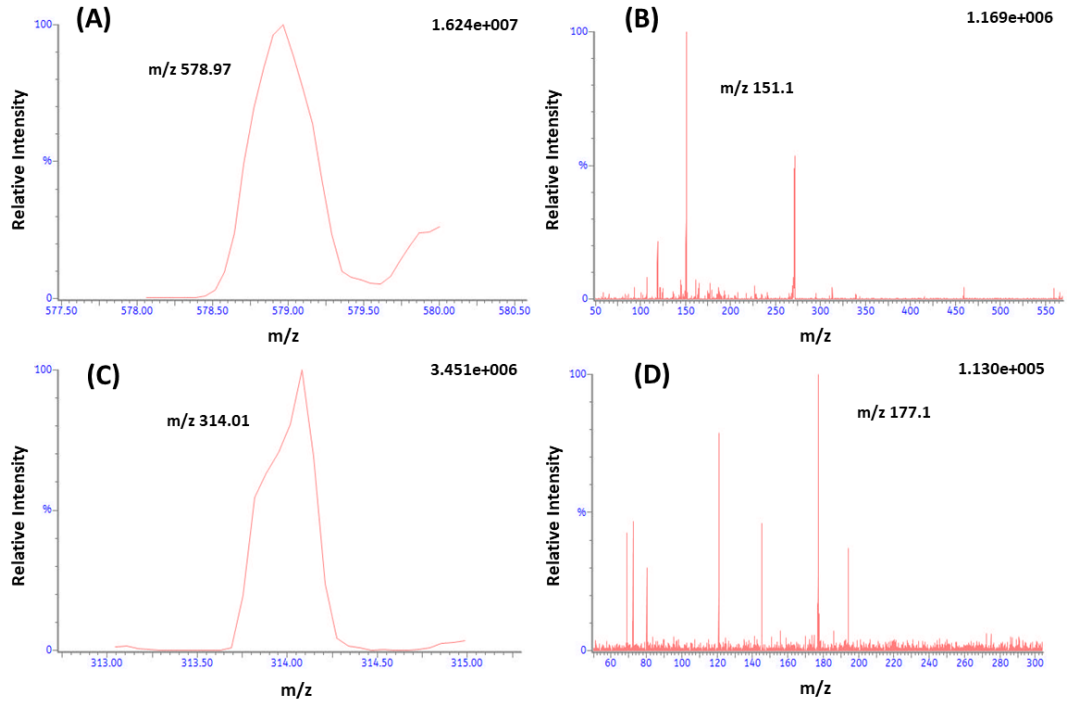

Figure 3. 16. Molecular ion and product ion selection from optimization process for (A, B) Naringin; (C, D) N-Feruloyltyramine.

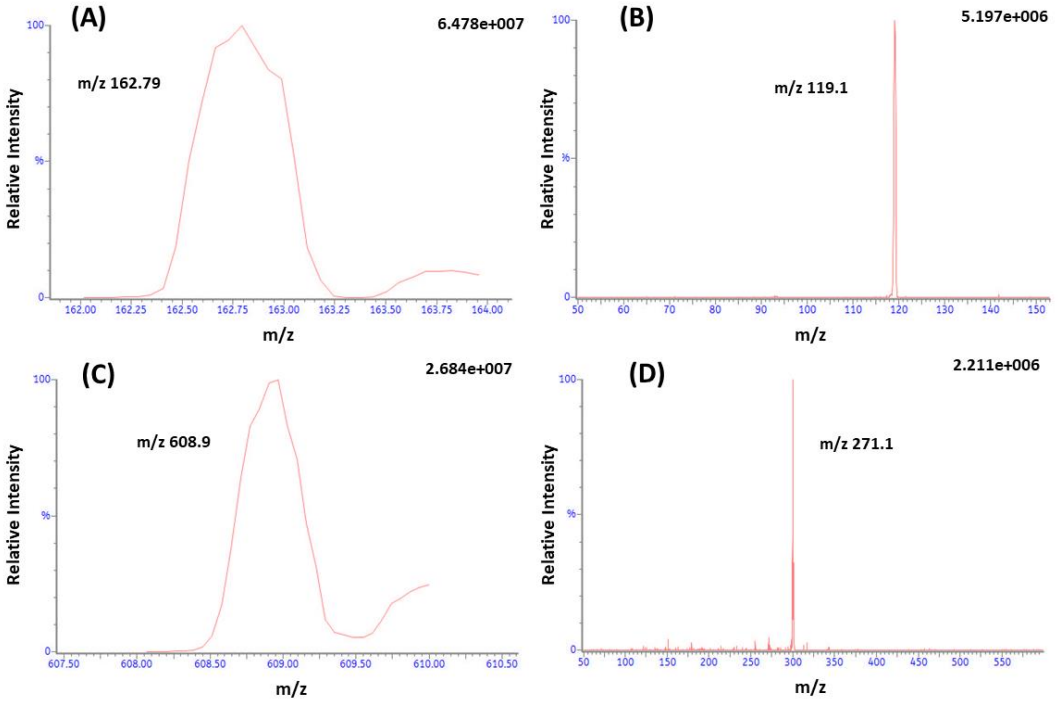

Figure 3. 17. Molecular ion and product ion selection from optimization process for (A, B) p-Coumaric acid; (C, D) Rutin.

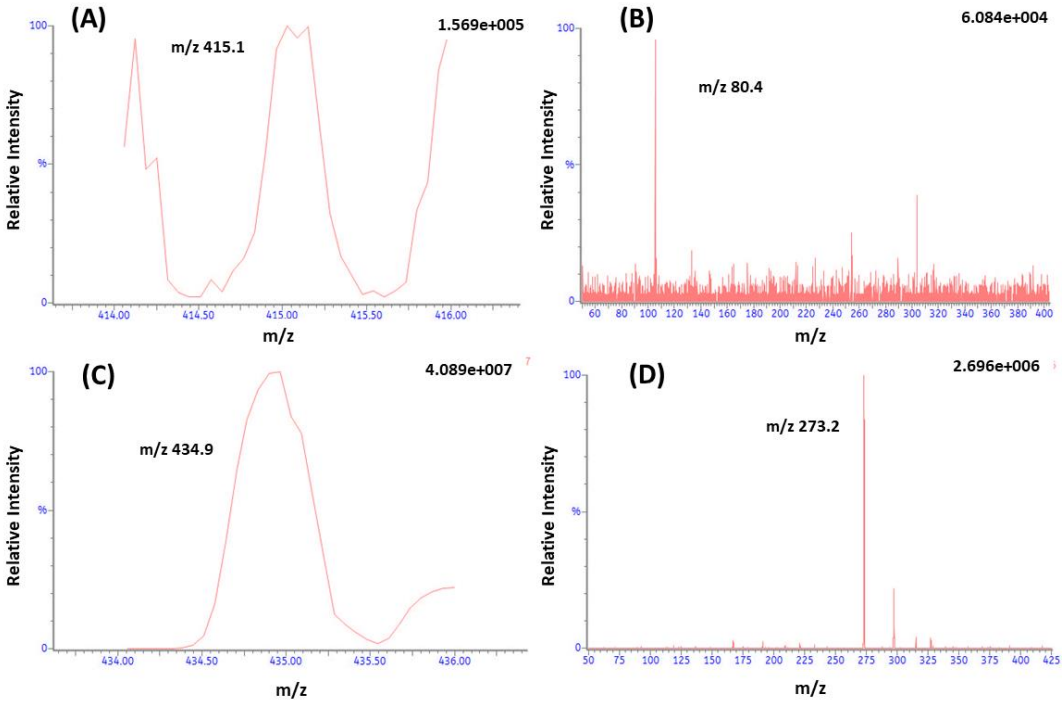

Figure 3. 18. Molecular ion and product ion selection from optimization process for (A, B) Sitosterol; (C, D) Trilobatin.

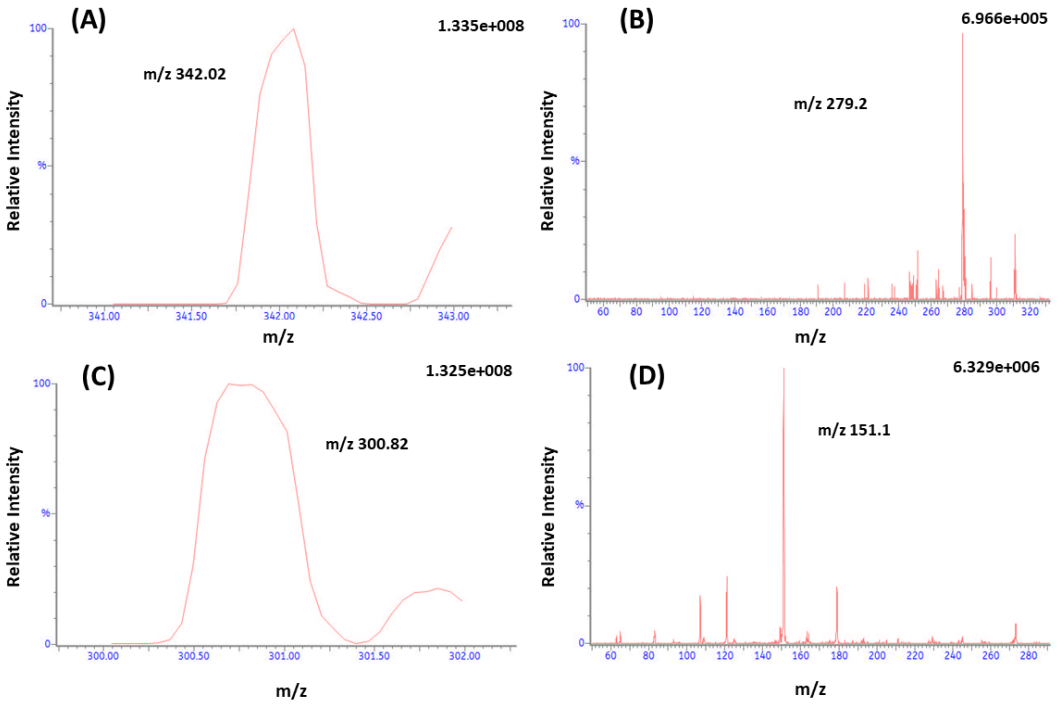

Figure 3. 19. Molecular ion and product ion selection from optimization process for (A, B) Isocorydine; (C, D) Quercetin.

Figure 3.20 shows extracted MRM chromatograms for four compounds found cultivar 22. In order to quantify the targeted compounds, standard curves were created by injected known concentrations (standards) of each compound (**Figure 3.21 and Figure 3.22**).

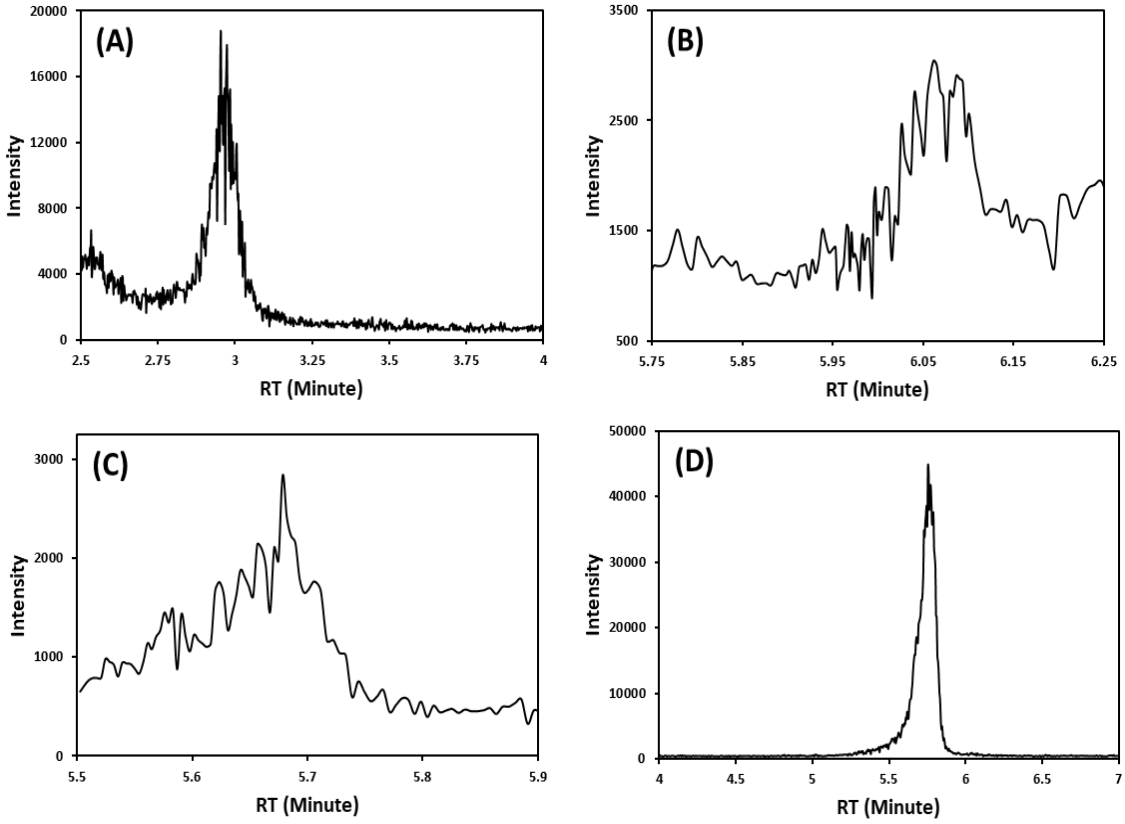

Figure 3. 20. Extracted MRM chromatograms for target compounds found in cultivar 22. (A) Citric acid; (B) (±)-Catechin; (C) 3,4-Dihydroxybenzoic acid; (D) Epigallocatechin.

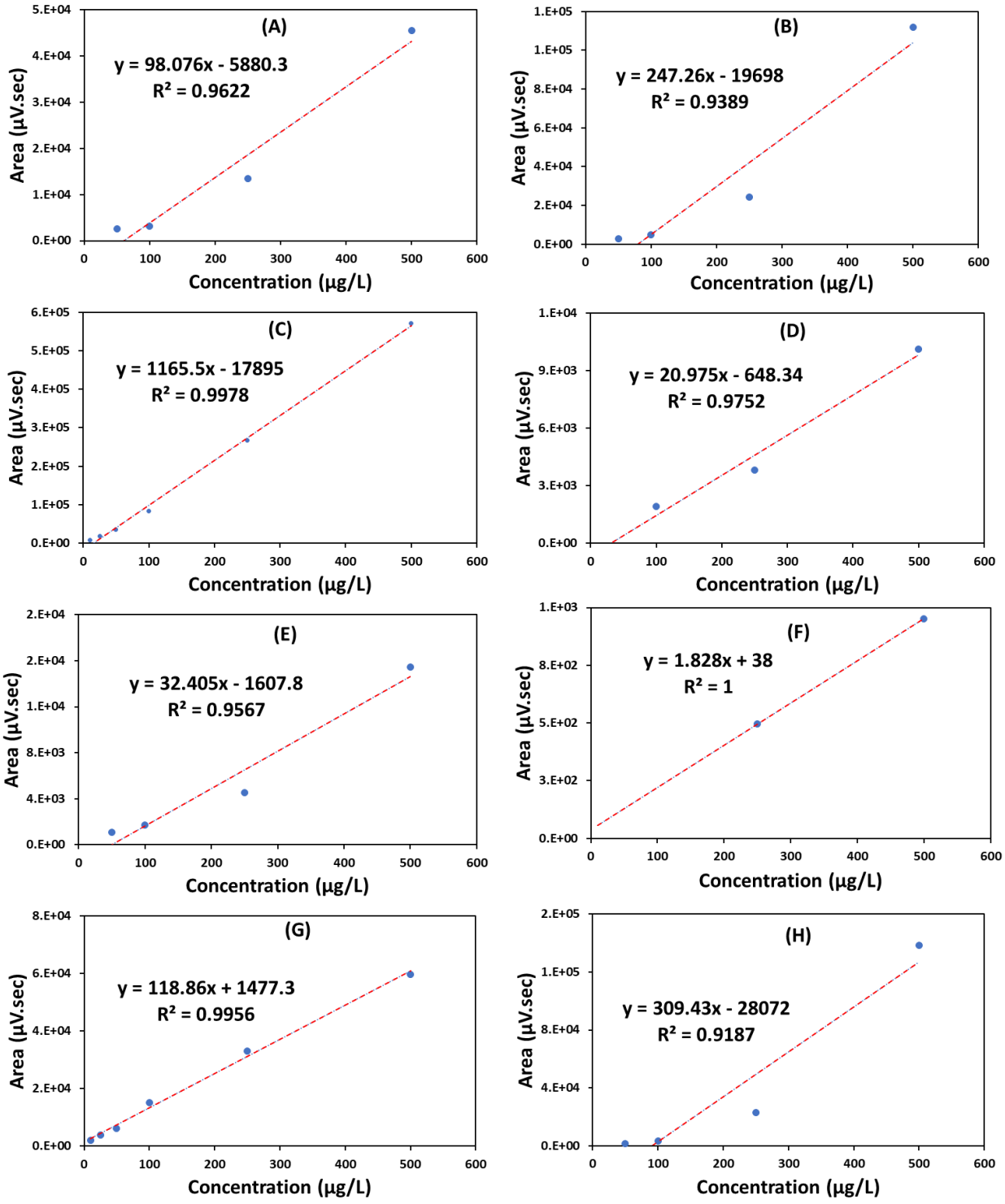

Figure 3. 21. Standard curves for (A) Catechin; (B) 3,4-Dihydroxybenzoic acid; (C) Caffeic Acid; (D) Citric acid. (E) Epicatechin; (F) Epigallocatechin; (G) Ferulic acid; (H) Gallocatechin 3-O-gallate.

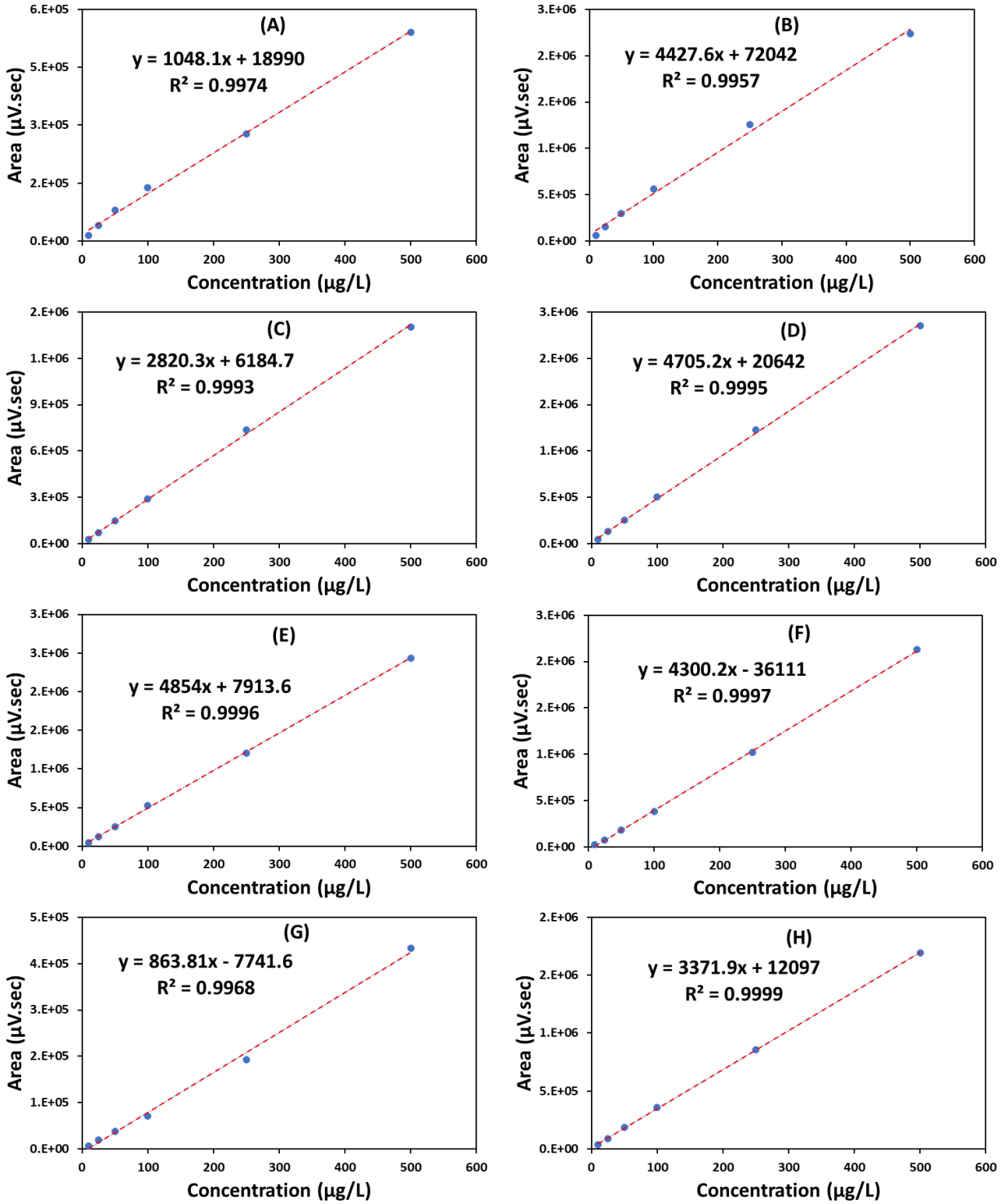

Figure 3. 22. Standard curves for (A) Genistein; (B) Isocorydine; (C) Naringin; (D) N-Feruloyltyramine; (E) p-Coumaric acid; (F) Quercetin; (G) Rutin; (H) Trilobatin.

The concentration of bioactive compounds in each cultivar was summarized in **Table 3.3**. The concentration of citric acid ranged between 8.55±2.2 mg/Kg to 519.72±130 mg/Kg. Pande and Akoh (2010) found the concentration of citric acid 100.6±40.6, 160.2±70.3, 180.7±100.5, and 90.8±40.9 mg/Kg in seed, pulp, peel and leaf, respectively. Citric acid had a significant difference between the cultivars with p= 0.005. Epigallocatechin, a phenolic compound, had a concentration between 1.29±0.4 mg/Kg and 5.11±1.5 mg/Kg. Nam et al. (2019) found the concentration of epigallocatechin in the ripe pawpaw 130.82±3.3 mg/Kg. Epigallocatechin had a significant difference between the cultivars with p= 0.008.

De Moraes et al. (2020) determined the concentration of different phenolic compounds in North American pawpaw. The concentration of 3,4-Dihydroxybenzoic acid in ripe pawpaw was found to be 11.2±0.07 mg/Kg which is consider higher than the concentration in this study (0.31±1.7 – 0.46±0.02) (p=0.05). Catechins are a type of natural plant compound belonging to the flavonoid family. They are widely recognized for their antioxidant properties and health benefits. Catechins are commonly found in various plant-based foods and beverages, with tea leaves, particularly green tea, being one of the most well-known sources (De Moraes et al., 2020). The concentration of catechin ranged between 0.29±0.1 and 1.37±0.84 mg/Kg (p= 0.063). Nam et al. (2019) found the concentration of catechin in the unripe pawpaw1420.25±7.9 mg/Kg. Catechin could be detected in the study conducted by (De Moraes et al., 2020). These variations could result from instrument sensitivity and/or complex physiological and biochemical changes that occur during the fruit's maturation process (ripe vs unripe).

Epicatechin is a type of flavonoid, which is a subclass of polyphenolic compounds found in various plant-based foods. It belongs to the catechin family of flavonoids and is commonly found in foods such as tea, cocoa, certain fruits, and vegetables. Epicatechin is known for its potential health benefits, particularly its antioxidant and cardiovascular properties. The concentration of epicatechin ranged between 0.47±0.15 mg/Kg and 3.01±0.5 mg/Kg (p= 0.031). Isocorydine is an alkaloid compound found in certain plant species. It belongs to the larger group of alkaloids, which are naturally occurring organic compounds that often have pharmacological effects (Avula et al., 2018). The concentration of isocorydine ranged between <LOD to 0.05±0.01 mg/Kg.

The concentration of caffeic acid in pawpaw can vary based on factors such as the variety of pawpaw, growing conditions, ripeness, and the specific part of the fruit being analyzed (e.g., skin, flesh, seeds). In general, pawpaw is not known to be an exceptionally high source of caffeic acid compared to some other fruits and vegetables. However, it still contributes to the overall antioxidant and phytochemical content of the fruit. De Moraes et al. (2020) could not detect caffeic acid in the ripe fruit. However, in this study, the concentration ranged between <LOD to 0.12±0.03 mg/Kg (p= 0.48).

Rutin is a flavonoid, which is a type of plant compound that belongs to the polyphenol group, found in various plant-based foods, and it's recognized for its potential health benefits. The concentration of rutin ranged between 0.34±0.03 mg/Kg to 1.08±0.02 mg/Kg (p= 0.002). Different study determined that the concentration of rutin was 1.63 ± 0.05 mg/Kg in the ripe pawpaw. However, Nam et al. (2019) found the concentration of rutin in the ripe pawpaw 2970.06 ±730.16 mg/Kg in unripe pawpaw.

p-Coumaric acid is a type of hydroxycinnamic acid, which is a subclass of phenolic compounds found in various plant-based foods. It is known for its potential health benefits and plays a role in the flavor, aroma, and color of certain foods. Nam et al. (2019) observed a difference in concentrations between ripe and unripe fruit. The concentrations were 220.07 ± 9.8 and 550.72 ± 20.31 mg/Kg in the ripe and unripe pawpaw, respectively. De Moraes et al. (2020) found the concentration of p-Coumaric 3.89 μg/g. In this study the concentration ranged between 0.002 to 0.09 mg/Kg (p= 0.004). Different varieties or cultivars of pawpaw may have varying levels of p-coumaric acid. Just like other plants, different pawpaw varieties can have genetic variations that impact their chemical composition. Furthermore, the environmental conditions in which pawpaw fruits are grown can influence their phytochemical content, including p-coumaric acid. Factors such as soil type, climate, altitude, and weather patterns can all play a role. The stage of ripeness of the pawpaw fruit can affect its phytochemical composition, including p-coumaric acid. Some compounds may increase or decrease as the fruit ripens. Other factors like temperature, humidity, and storage duration can affect the stability of compounds.

Ferulic acid is another type of hydroxycinnamic acid, similar to p-coumaric acid, that is found in various plant-based foods. The concentration of ferulic acid ranged between <LOD to 1.7±0.4 mg/Kg (p= 0.035). Nam et al. (2019) compared the concentration of ferulic acid between ripe and unripe pawpaw. The result showed that the concentration of ferulic acid in ripe pawpaw was 120.87±10.23 mg/Kg compared to 910.98 ± 60.86 mg/Kg in unripe pawpaw. Quercetin is a flavonoid compound that is found in various plant-based foods. Quercetin can be detected in pawpaw fruit, but its

concentration is relatively low compared to other fruits and vegetables that are known for their higher quercetin content. For example, in this study, the concentrations in all cultivars were lower than the instrument detection limits. This finding concur with De Moraes et al., (2020). Finally, naringin could not be detected in this study. However, Nam et al. (2019) found the concentration of naringin 370.80 ± 70.38 mg/Kg in the unripe pawpaw.

Table 3. 3. The concentration of bioactive compounds in each cultivar.

	Average Concentration (mg/Kg)							
Compound	**10-35**	**Overleese**	**PA Golden**	**Shenandoah**	**Sunflower**	**Susquehanna**	**Wells**	**Wilson**
Citric acid	8.55±2.2	11.28±2.5	519.72±130	50.84±17	483.92±140	13.24±3.7	402.92±142	382.85±130
Epigallocatechin	3.07±1.7	ND	1.61±0.5	1.29±0.4	ND	5.11±1.5	ND	ND
3,4-Dihydroxybenzoic acid	0.31±1.7	ND	0.4±0.04	ND	ND	0.46±0.02	ND	ND
(±)-Catechin	0.45±0.15	0.29±0.1	0.53±0.2	0.56±0.63	1.07±0.4	0.91±0.2	0.62±0.01	1.37±0.84
Epicatechin-(-)	0.47±0.15	0.36±0.1	0.84±0.2	0.72±0.2	1.19±0.6	2.4±0.4	0.94±0.01	3.01±0.5
Isocorydine (+)	ND	ND	ND	0.05±0.01	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01
Caffeic Acid	ND	ND	0.07±0.02	0.1±0.01	0.07±0.04	0.12±0.03	ND	ND
Rutin	0.34±0.03	0.18±0.01	0.94±0.02	1.08±0.02	0.6±0.01	0.31±0.11	0.9±0.05	0.66±0.04
p-Coumaric acid	0.002	0.002	ND	0.02±0.01	0.03±0.01	0.9±0.05	0.01±0.01	0.09±0.01
Ferulic acid	ND	ND	0.31±0.01	1.7±0.4	ND	ND	0.54±0.01	ND
Trilobatin	ND	ND	0.02±0.01	0.02±0.01	ND	ND	ND	ND
Quercetin	0.02±0.01	0.01±0.01	0.01±0.01	0.02±0.01	0.02±0.01	0.03±0.01	0.02±0.01	0.02±0.01
Naringin	ND	ND	ND	ND	ND	ND	ND	ND
N-Feruloyltyramine	ND	ND	ND	ND	ND	ND	ND	ND
Gallocatechin 3-O-gallate	ND	ND	ND	ND	ND	ND	ND	ND
Genistein	ND	ND	ND	ND	ND	ND	ND	ND

ND: not detected

3.4 Conclusion

LC-MS/MS methods were developed for quantification of bioactive compounds in pawpaw fruit. The analytes were analyzed in positive and negative ionization modes, which provided good sensitivity and selectivity, with detection limits ranging from 1.3 µg/L to 556.4 µg/L. The concentrations of the compounds ranged between 0.02±0.01 to 519.72±130 mg/Kg.

CHAPTER 4: PROFILING BIOACTIVITY OF PAWPAW COMPOUNDS PRESENT USING A HIGH-THROUGHPUT SCREENING APPROACH

4.1 Introduction

Asimina triloba (L.) Dunal, commonly known as pawpaw or “poor man's banana”, is the only member of the Annonaceae family growing in temperate zones and in particular grows wild in the eastern United States, ranging from northern Florida to southern Ontario (Canada) and as far west as eastern Nebraska (Lolletti et al., 2021). Pawpaws are very nutritious fruits containing significant amounts of vitamin A and C, and high levels of health promoting phenolics such as riboflavin and niacin (Grygorieva et al., 2021). Pawpaw trees produce natural compounds (annonaceous acetogenins) in leaf, bark, and twig tissues that possess both highly antitumor and pesticidal properties. Traditionally, American pawpaw has been used for treating fever, vomiting, and pain and swelling (inflammation) of the mouth and throat. In addition, the fruit has been used as a laxative, leaves are diuretic, and are applied externally to boils, ulcers and abscesses (Callaway, 1992; Grygorieva et al., 2021).

The roots, twigs, and seeds of pawpaw contain acetogenins, which are strong inhibitors of cancer cells. Pawpaw leaf essential oil has also been shown to have strong activity against cancer cell lines (Ko et al., 2011a; Nam et al., 2017). The pawpaw fruit has a taste that resembles a combination of mango, pineapple, and banana, and is rich in nutritive components such as vitamins and minerals (Templeton et al., 2003). Furthermore, pawpaw contains procyanidins that possess antioxidant properties, as stated

by Brannan et al. (2015). Due to the increasing commercial significance of pawpaw, multiple studies have investigated various aspects of its physiology. For example, the physical, chemical, and sensory characteristics of pawpaw were described by (McGrath & Karahadian, 1994), while the lipids of the fruit were examined by (Wood & Peterson, 1999). Kobayashi et al. (2008) also demonstrated that the phenolic content and antioxidant capacity of pawpaw fruit varies depending on the stage of ripeness. Brannan et al. (2015) analyzed the phytochemical content and quality characteristics of ten different varieties of pawpaw fruit pulp. Additionally, Ortutu et al. (2015) evaluated the antioxidant activity of various tropical fruits, including mango, pawpaw, and guava, at different stages of maturation. However, most previous studies have focused solely on edible fruit, resulting in a lack of information regarding other plant tissues such as roots, twigs, and leaves. Furthermore, there have been no studies conducted on the phenolic acid compounds found in pawpaw.

High-throughput screening (HTS) is a critical tool to expand biomedical knowledge of small molecules that can be used for the drug discovery industry (Macarron et al., 2011). The high-throughput analytical technologies enable us to evaluate the biological functions of large amounts of chemicals or natural materials in the shortest amount of time by integrating chemical analyses, modeling, and machine learning that can result in marketed pharmaceutical products in the lowest cost production (Leavell et al., 2020). In this study, we utilized high-throughput screening assays to identify antioxidant, antibacterial and anticancer potentials of the compounds found in pawpaw fruit. The exploration could reveal underexplored bioactive activities of pawpaw extracts and promote the development of novel applications of pawpaw and its by-products.

4.2 Objectives

In this study, we utilized high-throughput screening assays to identify antioxidant, antibacterial and anticancer potentials of the compounds found in pawpaw fruit. The exploration of biological functions of bioactive compounds in pawpaw could reveal underexplored bioactive activities of fruit extracts and promote the development of novel applications of pawpaw and its by-products. The specific objectives of this research:

1- Test Total Antioxidant Capacity (CUPRAC).
2- Test the Activation of Antioxidant Response element (HepG2-ARE cells).
3- Test anticancer activity by exposing HL-60 (Leukemia Cell line), SKMEL5 (Melanoma cell line), MCF-7 (Breast Cancer Cell line), SKOV3 (Ovarian Cancer Cell line) and PC3 (Prostate Cancer Cell line) cells to pawpaw compounds.
4- Test antibacterial activity by exposing Mycobacterium smegmatis, Pseudomonas aeruginosa, E. coli and Staphylococcus aureus to pawpaw compounds.

4.3 Materials and Methods

4.3.1 Cell Lines

A liver cell line, HepG2-ARE, which expresses a firefly luciferase gene under the control of an Nrf2 antioxidant response element (ARE), was purchased from BPS Bioscience in San Diego, CA. Additionally, two other human cell lines, A549 (alveolar epithelial cells) and MRC-5 (lung fibroblast cells), were obtained from the American Type Culture Collection in Virginia. The HepG2-ARE cells were cultivated in modified Eagle's medium supplemented with GlutaMAX, 10% fetal calf serum, and 600ug/mL Geneticin from Thermo Fisher Scientific, while the A549 and MRC-5 cells were

cultivated in RPMI medium with 10% FBS. All cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

4.3.2 Total Antioxidant Capacity

In order to determine the antioxidant capacity of the compounds, a colorimetric assay kit (K274-100, BioVision, CA, USA) was used following the manufacturer's instructions. The compounds were tested at eight different concentrations and added to 384-well plates, and then a Cu^{2+} working solution was added to the sample wells. After incubating the plates for 1.5 hours at room temperature, the absorbance of the samples was read at 570 nm using a microplate reader (Enspire, Perkin Elmer Inc., Waltham, MA, USA). To standardize the antioxidant capacity, Trolox was used as recommended by the manufacturer, and a Trolox standard curve was included. The total antioxidant capacity of the compounds was calculated using curve-fitting software and expressed as Trolox equivalent (mM) from an eight-parameter logistic curve of the Trolox control.

4.3.3 Antioxidant Response Element (ARE) Activation

The effect of the compounds on the activation of ARE in the HepG2-ARE cell line was assessed using the Steady-Glo® Luciferase assay system (E2510, Promega, Madison, WI, USA) following the manufacturer's instructions. First, the HepG2-ARE cells were seeded in 384-well plates with 50 µL of complete media per well at a density of 10,000 cells/well using a Multidrop Combi dispenser (Thermo Fisher Scientific, Waltham, MA, USA), and then the plates were incubated at 37 °C in a 5% CO_2 humidified incubator for 20 hours. The HepG2-ARE cells were then incubated with the

compounds for 18 hours, with the known ARE activator TBHQ used as a positive control, and the cells treated with 0.35% DMSO and without the tested compounds used as a vehicle control. Cells without DMSO and compounds were used to measure background luminescence. Reporter activity was measured by adding 25 µL of Steady-Glo® luciferase assay reagent (Promega) for 30 minutes using the Multidrop Combi dispenser (Thermo Fisher Scientific), and luminescence intensities were read using an Enspire microplate reader (Perkin Elmer Inc.). The percentage cytotoxicity of the compounds was normalized to the positive and negative controls on each assay plate.

4.3.4 Anti-Cancer Activity

HL-60 (Leukemia Cell line), SKMEL5 (Melanoma cell line), MCF-7 (Breast Cancer Cell line), SKOV3 (Ovarian Cancer Cell line) and PC3 (Prostate Cancer Cell line) cells were exposed to the pawpaw compounds for 72 hours at 37°C, 5% CO_2 in a 95% humidified incubator followed by the addition of the Cell Titer-Glo Luminescent assay reagent (Promega, Madison, WI). The plates were shaken for 2 minutes at 1000 rpm and luminescence was read 20 min later on a Tecan Safire2 microplate reader (Mannedorf, Switzerland). Percent cytotoxicity was normalized to DMSO and no cell controls. Non-linear regression analysis of the dose-response data was performed using GraphPad Prizm and EC50 or concentrations for 50% cytotoxicity were reported for each compound.

4.3.5 Anti-Bacterial Activity

The activity of A. triloba compounds was tested against Staphylococcus aureus, subsp. aureus Rosenbach (ATCC 29213), Pseudomonas aeruginosa (Schroeter) Migula PAO1 (ATCC 15692) and Escherichia coli (Migula) Castellani and Chalmers (ATCC 25922). The bacteria from a single colony were grown overnight in Mueller Hinton cation-adjusted broth at 37 °C with constant agitation at 250 rpm. The overnight culture was diluted 100-fold, and 40 µL of bacterial suspension was grown in the presence of the compounds diluted to seven concentrations, double-diluted in DMSO. The test plates were incubated for 24 h at 37 °C. The activity of compounds was also evaluated against Mycobacterium smegmatis, which was grown in Middlebrook 7H9 OADC supplemented media at 37 °C, 250 rpm, for 24 h & 48 h. Positive controls included vancomycin and amikacin, while DMSO served as the negative control in each assay plate. The plates were read at an absorbance of 600 nm after 24 h and 48 h of incubation. After the absorbance reads, Bact-Titer Glo was added to the plates, and an ATP-based luminescence assay was read using a BioTek Synergy microplate reader. The percent inhibition of growth was normalized to the positive and negative controls on each assay plate.

4.3.6 Statistical and data analysis

For total antioxidant capacity analysis, linear regression analysis was performed to identify the linear regression equation for each compound using GraphPad Prism 8 software (San Diego, CA, USA). The coefficient of the compound equation was compared with the Trolox control's coefficient to determine each compound's relative

total antioxidant capacity. The fold increase over Trolox was calculated by dividing the compound models' coefficient by the Trolox control coefficient (Ho et al., 2020; Roy et al., 2019).

The ARE fold induction of the compounds was measured by dividing the luminescence absorbance of the treatment by the specific luminescence absorbance of the control sample and multiplying by 100. The control sample (in the presence of DMSO control and without the compounds) was set at 100% (Ho et al., 2020; Roy et al., 2019).

The phenolic compounds' relative cytotoxicity (%) was calculated as follows.

$$\%\ Cytoxicity = \frac{A}{B}\ x\ 100$$

Where A is the specific luminescence absorbance of the treated sample, and B is the specific luminescence absorbance of the control sample. The control sample (in the presence of the DMSO vehicle control and without the compounds) was set to 100% no cytotoxicity. Non-linear regression analysis of the data was performed to identify the dose-response curve for each compound. Each compound's 50% inhibitory concentration values (IC50) were determined from the dose-response curve for the cell lines using GraphPad Prism 8 software (Ho et al., 2020; Roy et al., 2019).

4.4 Results and Discussion

The A. triloba methanolic extract of the ripe fruit had a high concentration of phenolic compounds. Phenolic compounds are known for their antibacterial, anti-inflammatory and anticancer activity that could be linked to the traditional use of the plant. Studies have found high concentrations of phenolic compounds in the fruit, leaves, roots, and twigs of A. triloba (Nam et al., 2017, 2019b). However, studies on the phenolic

composition of A. triloba ripe fruit have not been fully explored, which is addressed in the present study. Further studies on the compound composition of the different plant parts of A. triloba could potentially identify novel compounds for treating various diseases.

4.4.1 Total antioxidant capacity (CUPRAC- assay)

The CUPRAC assay is a single electron transfer mechanistic assay that determines the reducing capacity of Cu (II) ions. Phenolic compounds that are a normal part of human nutrition play a crucial role in combating free radicals and oxidative stress linked to numerous maladies, including cancer and inflammation. Twenty-eight compounds were tested for their total antioxidant capacity compared to Trolox, an antioxidant analog of vitamin E. Fifteen compounds, namely (±)-catechin, (+)-syringaresinol, adriamycin, 3,4-dihydroxybenzoic acid, quercetin, rutin, gallic acid, caffeic acid, epicatechin, chlorogenic acid, epigallocatechin, (-)-catechin gallate, isocorydine, ferulic acid, and ascorbic acid had a higher total antioxidant capacity than Trolox (**Table 4.1**, **Figures 4.1 and 4.2**). Fourteen compounds had a statistically significant antioxidant fold increase higher than one over Trolox ($P < 0.01$), except for ascorbic acid ($P > 0.05$) (**Table 4.1**).

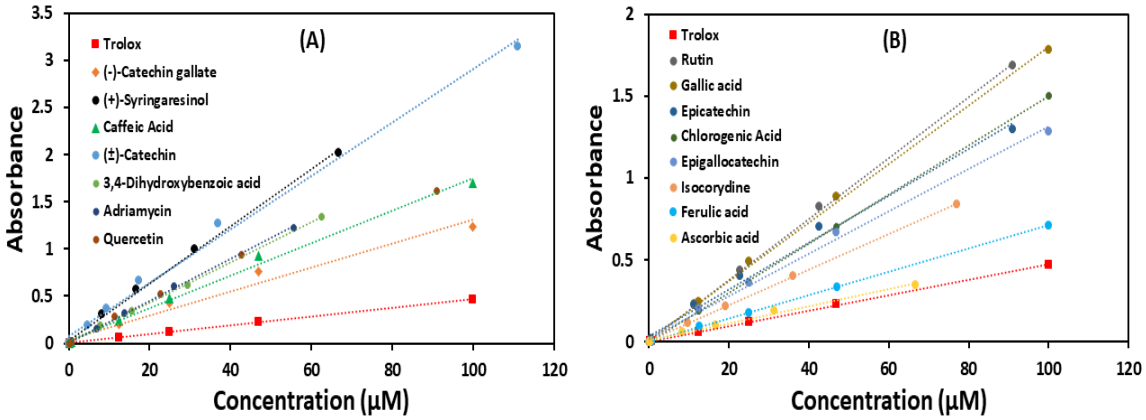

Figure 4. 1. Total antioxidant activity of the compounds in pawpaw fruit. (A, B) Compounds with higher antioxidant capacity than Trolox.

The higher antioxidant activity of quercetin, caffeic acid, and ferulic acid is in accordance with the studies conducted by Kalinowska et al. (2022). The high antioxidant capacity of quercetin is due to the hydroxy (-OH) groups in the 3'- and 4'- positions of the B ring and the number 3-position of the C- ring. Caffeic acid has a greater reducing capacity than ferulic acid due to the presence of two hydroxyl groups on the aromatic ring, as opposed to ferulic acid's methoxy group (R−O−CH3). Replacing a hydrogen atom with a methoxy group increases the compounds reducing capacity. However, replacing a hydroxyl group with a methoxy group reduces the antioxidant activity of a compound (**Table 4.1**). The higher total antioxidant capacity of ferulic acid than p-coumaric acid is due to the methoxy group in the ortho-position to the -OH group. The methoxy group increases ferulic acid's electron transfer capacity and the compound's stability to the methoxy group in the ortho-position (Kalinowska et al., 2022; Özyürek et al., 2011).

(+)-Catechin, a trans-configured isomer of (-)-epicatechin, an isomer with a cis-configured hydroxyl group in the third position of the C-ring, possessed a greater total

antioxidant capacity than epicatechin with the CUPRAC assay (**Table 4.1**). The CUPRAC results in the present study contrast with previous studies done by Ho et al. (2020). The researcher determined epicatechin to have a higher antioxidant capacity than catechin. This change is stability, and the total antioxidant capacity could be due to the variation in the methodologies. The total antioxidant capacity of the compound was measured by Ho et al. (2020) through the reduction of Cu(II) to Cu(I). The present study used Cu(Nc)2+ (Cu(II)- Neocuproine), which results in a stable Cu(Nc)2+ species compared to Cu(II)/Cu(I) without neocuproine to stabilize the complex resulting in a faster and complete redox reaction. The results of the CUPRAC assay are in accordance with the studies conducted by Özyürek et al. (2011) that developed the assay (Kalinowska et al., 2022; Nowak et al., 2018; Özyürek et al., 2011; Platzer et al., 2021). Several compounds tested displayed significant antioxidant activity higher than the positive control, Trolox. In food and medicinal plants, polyphenols and ascorbic acid act in synergy to have an increased free radical scavenging activity (Kalinowska et al., 2022; Nowak et al., 2018; Özyürek et al., 2011; Platzer et al., 2021). Therefore, the anti-inflammatory activity reported in Native American traditional medicine is likely due to the synergistic reaction between the polyphenolic compounds and ascorbic acid found in A. triloba ripe fruit.

Table 4. 1. Total antioxidant capacity of *Asimina triloba* compounds compared to Trolox.

Putatively identified compounds	Slope ± SD	R^2	Slope fold increase over Trolox
Trolox	$4.71x10^{-3} \pm 1.30x10^{-5}$	0.9998	1.00
(±)-Catechin	$3.45x10^{-2} \pm 1.42x10^{-4}$**	0.9958	7.34
(+)-Syringaresinol	$3.10x10^{-2} \pm 1.02x10^{-3}$**	0.9968	6.58
Adriamycin	$2.21x10^{2} \pm 1.21x10^{-4}$**	0.9994	4.69
3,4-Dihydroxybenzoic acid	$2.17x10^{-2} \pm 6.21x10^{-4}$**	0.9996	4.61
Quercetin	$1.87x10^{-2} \pm 9.82x10^{-4}$**	0.9899	3.97
Rutin	$1.85x10^{-2} \pm 1.60x10^{-4}$**	0.9990	3.92
Gallic acid	$1.79x10^{-2} \pm 4.19x10^{-5}$**	0.9992	3.80
Caffeic Acid	$1.78x10^{-2} \pm 7.13x10^{-4}$**	0.9959	3.04
Epicatechin	$1.47x10^{-2} \pm 5.15x10^{-4}$**	0.9911	3.13
Chlorogenic Acid	$1.43x10^{-2} \pm 9.07x10^{-4}$**	0.9984	3.04
Epigallocatechin	$1.31x10^{-2} \pm 2.84x10^{-4}$**	0.9975	2.79
(-)-Catechin gallate	$1.27x10^{-2} \pm 3.47x10^{-5}$**	0.9782	2.70
Isocorydine	$1.05x10^{-2} \pm 4.80x10^{-4}$**	0.9983	2.24
Ferulic acid	$6.97x10^{-3} \pm 2.42x10^{-4}$**	0.9997	1.48
Ascorbic acid	$5.92x10^{-3} \pm 8.92x10^{-4}$	0.9940	1.26
p-Coumaric acid	$3.99x10^{-3} \pm 2.69x10^{-5}$	0.9932	0.85
Genistein	$3.57x10^{-3} \pm 2.10x10^{-4}$	0.9649	0.76
Naringin	$4.71x10^{-4} \pm 2.26x10^{-5}$	0.9930	0.10
Citric acid	$1.64x10^{-4} \pm 2.75x10^{-4}$	0.0945	0.03
Annonacin	$5.44x10^{-5} \pm 1.66x10^{-5}$	0.0817	0.01
Tartaric acid	$3.28x10^{-5} \pm 2.65x10^{-5}$	0.6976	0.01
β-Sitosterol	$1.38x10^{-5} \pm 6.94x10^{-5}$	0.2340	0.00
Malic acid	$6.76x10^{-6} \pm 2.11x10^{-5}$	0.3880	0.00
Cinnamic acid	$5.87x10^{-7} \pm 8.40x10^{-6}$	0.0415	0.00
Artemisinin	$-5.88x10^{-6} \pm 7.09x10^{-6}$	0.0615	0.00
Quinic acid	$-7.65x10^{-6} \pm 8.03x10^{-6}$	0.0777	0.00

***** P < 0.001, ** P < 0.01 and * P < 0.05: statistically significant difference to the positive control; P > 0.05 no statistically significant difference to the positive control. One-way ANOVA followed by Dunnets t-test against Trolox, n=3.**

4.4.2 Antioxidant Response Element (ARE) activation

The NrF2 transcription factor binds to antioxidant response elements in response to free radicals. The Nrf2/ARE pathway is involved in the gene regulation of genes encoding for antioxidant enzymes and detoxifying proteins in the human body, preventing oxidative damage (Roy et al., 2019; Vomhof-DeKrey & Picklo, 2012). Tert-butylhydroquinone (tBHQ) is an activator of the Nrf2/ARE pathway and served as a positive control in the present study. Adriamycin and quercetin at $0.1 \pm 4.2x10^{-3}$ µM and $0.3 \pm 5.5x10^{-3}$ µM displayed no statistically significant difference in activation of the

Nrf2/ARE pathway compared to tBHQ, respectively ($P > 0.05$) (**Table 4.2**). It has been discovered that quercetin has chemopreventive effects against various malignant cell lines and temporarily activates the Nrf2/ARE pathway at low concentrations, which agrees with the present study. The Nrf2/ARE pathway is activated in the presence of quercetin as a protective effect from exposure to cytotoxic flavonoids. It has been proposed that the inhibition of the p38-MAPK signaling pathway and activation of the Nrf2/ARE pathway by quercetin provides temporary protection against oxidative and inflammatory cell damage (Granado-Serrano et al., 2012; Kraft et al., 2004).

Adriamycin is a chemotherapeutic agent used in the treatment of breast cancer. The present *in vitro* study determined that adriamycin activates the NRF2/ARE pathway. However, mice studies have confirmed that adriamycin results in a Ca^{2+} influx and increase in mitochondrial superoxide in epithelial cells, resulting in an overproduction of reactive oxygen species. An increase in reactive oxygen species inhibits protein kinase B and the NRF2/ARE pathway *in vivo* (Wang et al., 2015). As Wang et al. (2015)discussed, the ARE assay is a powerful tool for discovering novel compounds as potential anti-inflammatory and anticancer therapies. However, the limitation of this assay is that it does not consider the interactions of the compounds with multiple pathways that can affect the activation or inhibition of the NRF2/ARE pathway, which can only be observed in a complete *in vivo* system.

Table 4. 2. Antioxidant Response Element activation of *Asimina triloba* putatively identified compounds compared to the positive control tert-butylhydroquinone (tBHQ)

Putatively identified compounds	AC_{50} (μM) Mean ± SD	P-value
tBHQ	2.3 ± 0.6	-
(+)-Syringaresinol	30.4 ± 2.7	P < 0.01
Adriamycin	$0.1 \pm 4.2 \times 10^{-3}$	P > 0.05
Quercetin	$0.3 \pm 5.5 \times 10^{-3}$	P > 0.05
Ascorbic acid	33.0 ± 3.8	P < 0.01
Genistein	9.1 ± 0.1	P < 0.01
Cinnamic acid	66.1 ± 1.5	P < 0.01
Artemisinin	99.5 ± 0.7	P < 0.01

P < 0.001, P < 0.01 and P < 0.05: statistically significant difference to the positive control; P > 0.05 no statistically significant difference to the positive control. One-way ANOVA followed by Dunnets t-test against tBHQ, n =3.

4.4.3 Cell proliferation assay

The putatively identified compounds of *A. triloba* were tested against four cancer cell lines related to leukemia (HL-60), melanoma (SKMEL5), breast cancer (MCF-7), ovarian cancer (SKOV3), and prostate cancer (PC3). The samples were normalized to the vehicle control that served as the 100% cellular growth control. Secondary metabolites are classified as having significant (IC_{50} < 10 μM), moderate (10 μM < IC_{50} < 50 μM), low (50 μM < IC_{50} < 250 μM), and no cytotoxic (IC_{50} > 250 μM) activity against cancerous cell lines (Kuete & Efferth, 2015). Adriamycin and annonacin had the most significant anticancer activity against all five cell lines tested. All other compounds displayed moderate to low anticancer activity against one to two cancer cell lines (**Table 4.3**). Adriamycin is widely used in cancer therapies to treat leukemia and breast cancer, to name but a few. Adriamycin damages cellular DNA and inhibits enzymes responsible for cell division and DNA repair. Annonacin, an annonaceous acetogenin, has previously been effective against breast, bladder, and lung cancer and causes apoptotic cell death and extracellular signal-regulated kinase inhibition in these cancer cell lines (Yap et al.,

2017). Studies have determined that the unripe fruit of *A. triloba* has a higher concentration of acetogenins than ripe fruit, resulting in a higher antiproliferative effect on human cancer cell lines. The US National Cancer Institute defines antiproliferation as inhibiting cellular growth and cytotoxicity, the ability of a compound to cause cell death. The antiproliferative and cytotoxic studies previously observed for annonacin against MCF-7 cell lines align with the results observed in the present study (Ko et al., 2011b; Nam, Park, et al., 2018; Twilley et al., 2020). This study aimed to investigate the ripened fruit of *A. triloba*. Based on existing literature, the methanolic extract from the ripe fruit is anticipated to be less toxic than the unripe fruit. These results are consistent with the historical practice of consuming ripe fruit in moderation due to its lower acetogenin concentration.

Table 4. 3. Cytotoxicity of *Asimina triloba* secondary metabolites against leukemia (HL-60), melanoma (SKOV3), breast cancer (MCF-7), ovarian (SKMEL5) and prostate cancer (PC3) cell lines

Putatively identified compounds	$IC_{50}^{c} \pm SD^{d}$ (µM)				
	HL-60	**SKOV3**	**MCF-7**	**SKMEL5**	**PC3**
(-)-Catechin gallate	79.9 ± 3.6	110.7 ± 5.4	210.0 ± 14.1	NI[a]	82.2 ± 1.3
(+)-Syringaresinol	59.5 ± 0.4	NI[b]	200.0 ± 0.0	NI[b]	150.0 ± 0.0
(±)-Catechin	117.4 ± 8.8	NI[b]	75.0 ± 0.0	NI[b]	107.6 ± 3.5
3,4-Dihydroxybenzoic acid	150.0 ± 0.0	NI[b]	150.0 ± 0.0	NI[b]	180.0 ± 0.0
Adriamycin	$16.2x10^{-4} \pm 3.6x10^{-4}$	$6.8x10^{-2} \pm 6.6x10^{-2}$	$7.1x10^{-2} \pm 4.9x10^{-3}$	$8.6x10^{-2} \pm 1.7x10^{-3}$	$1.3x10^{-1} \pm 4.5x10^{-3}$
Annonacin	$4.5x10^{-1} \pm 7.0x10^{-2}$	$7.1x10^{-1} \pm 1.9x10^{-2}$	$5.1x10^{-1} \pm 5.4x10^{-2}$	$2.5x10^{-1} \pm 2.6x10^{-2}$	$2.0x10^{-1} \pm 4.2x10^{-2}$
Artemisinin	157.5 ± 10.6	NI[a]	200.0 ± 0.0	222.5 ± 3.5	122.5 ± 3.5
Ascorbic acid	37.1 ± 0.9	NI[b]	43.0 ± 0.9	210.6 ± 13.9	86.9 ± 4.4
Caffeic Acid	98.7 ± 9.0	NI[a]	201.4 ± 2.0	NI[a]	NI[a]
Chlorogenic Acid	NI[a]	NI[b]	200.0 ± 0.0	NI[a]	NI[a]
Cinnamic acid	NI[a]	NI[b]	NI[a]	NI[b]	NI[b]
Citric acid	220.0 ± 0.0	NI[b]	NI[a]	NI[b]	NI[b]
Epicatechin	151.2 ± 1.7	NI[a]	235.0 ± 21.2	230.0 ± 7.1	242.5 ± 3.5
Epigallocatechin	15.9 ± 0.4	79.8 ± 3.2	23.8 ± 0.8	82.7 ± 7.6	77.9 ± 2.9
Ferulic acid	127.5 ± 3.5	NI[b]	NI[a]	NI	NI[a]

Gallic acid	14.8 ± 0.9	89.4 ± 0.8	28.0 ± 0.2	246.5 ± 5.0	115.7 ± 6.4
Genistein	22.2 ± 2.1	79.3 ± 6.6	68.6 ± 0.5	80.4 ± 10.1	81.7 ± 8.8
Isocorydine	127.5 ± 3.5	NI[b]	195.0 ± 0.0	230.0 ± 0.0	212.5 ± 24.7
Malic acid	NI[a]	NI[b]	NI[a]	NI[b]	NI[b]
Naringin	NI[a]	NI[b]	NI[a]	NI[b]	NI[b]
p-Coumaric acid	NI[b]	NI[b]	NI[b]	NI[b]	181.8±0.0
Quercetin	30.4 ± 0.5	NI[a]	104.3 ± 8.1	102.8 ± 3.1	105.1 ± 1.4
Quinic acid	181.8 ± 0.0	NI[b]	181.8 ± 0.0	NI[b]	NI[b]
Rutin	NI[a]	NI[b]	NI[a]	NI[b]	NI[b]
β-Sitosterol	NI[a]	NI[b]	NI[a]	NI[b]	NI[a]
Tartaric acid	NI[a]	NI[b]	NI[a]	NI[b]	NI[b]

a) NI: IC_{50} above 250 µM to 300 µM; b) No inhibition at the highest concentration tested; c) IC_{50}: 50% inhibitory concentration; d) SD: standard deviation.

4.4.4 Antibacterial assay

Antimicrobial agents exhibit a range of potential modes of action, including (1) disruption of the cell membrane, (2) inhibition of cell wall synthesis, (3) inhibition of protein synthesis, (4) inhibition of nucleic acid synthesis, and (5) inhibition of the metabolic pathway (Reygaert, 2018). The studies conducted by Nam et al. (2019) emphasized the antibacterial activity of the ripe fruit of *A. triloba* compared to the unripe fruit. This observation is consistent with the present study's findings on the ripe fruit (**Table 4.4**)**.** Eleven compounds exhibited antibacterial activity against *P. aeruginosa*, with six compounds demonstrating no statistically significant difference compared to the positive control vancomycin ($P > 0.05$). The antimicrobial activity of quercetin against *P. aeruginosa* and *S. aureus* is attributed to its ability to disrupt the cellular membrane and inhibit nucleic acid synthesis in the bacteria, as indicated by IC_{50} values of 0.44 ± 0.22 µM and 36.0 ± 4.0 µM, respectively (**Table 4.4**) (Nguyen & Bhattacharya, 2022). Similarly, based on the literature, it can be hypothesized that epicatechin exerts its antimicrobial effect on *E. coli* by disrupting the cell membrane, as evidenced by an IC_{50} value of 0.15 ± 0.071 µM (**Table 4.4)** (Dias et al., 2021). Further investigation is

necessary to determine the specific antimicrobial mode of action for the remaining compounds that exhibited antibacterial activity (**Table 4.4**). The positive control amikacin and the compound adriamycin demonstrated noteworthy antibacterial activity against *S. aureus*, which was statistically comparable to vancomycin. None of the compounds demonstrated antibacterial activity against *M. smegmatis* that was not statistically different from the positive controls, vancomycin and amikacin (**Figure 4.2)**. Epicatechin and genistein displayed antibacterial activity toward *E. coli,* which was not statistically different from the positive control amikacin. The antibacterial activity observed for the ripe fruit could be related to the traditional use of the plant to treat skin boils and sores. The highest antibacterial activity was observed against bacteria associated with skin and wound infections, such as *P. aeruginosa* and *S. aureus*. Furthermore, the presence of *S. aureus* and *E. coli* as co-infectious microorganisms in sexually transmitted (STIs), gastrointestinal and urinary tract infections make the fruit of *A. triloba* an ideal candidate for further synergistic research of the compounds on these maladies based on the results in **Table 4.4**.

The World Health Organization recognized that microbial resistance research is of utmost importance and has prioritized it as a critical area of focus. Studies have confirmed that the bacteria investigated in this study can develop resistance to vancomycin, rendering it ineffective for treating infections caused by these bacteria (Ecevit et al., 2022). Further studies are necessary to assess the potential of *A. triloba* and its compounds in addressing microbial resistance by investigating their antibiofilm activity and quorum sensing inhibition.

Table 4. 4. Antibacterial of *Asimina triloba* secondary metabolites against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Mycobacterium smegmatis* and *Escherichia coli*.

Putatively identified compounds	IC_{50}[c] ± SD[d] (µM)			
	Pseudomonas aeruginosa	***Staphylococcus aureus***	***Mycobacterium smegmatis***	***Escherichia coli***
(-)-Catechin gallate	0.30 ± 0.017	NI[a]	NI[a]	NI[a]
(+)-Syringaresinol	0.48 ± 0.04	NI[a]	NI[a]	NI[a]
Adriamycin	0.11 ± 0.014	1.72 ±0.26	76.99 ± 5.08**	10.15 ± 0.42**
Annonacin	0.10 ± 0.002	17.46 ± 2.76**	300.0 ± 0.00**	NI[a]
Artemisinin	0.19 ± 0.0069	NI[a]	NI[a]	NI[a]
Caffeic Acid	13.8 ± 1.7**	NI[a]	NI[a]	NI[a]
Chlorogenic Acid	41.2 ± 4.6**	NI[a]	NI[a]	NI[a]
Epicatechin	NI[a]	NI[a]	NI[a]	0.15 ± 0.071
Genistein	5.40 ± 2.21**	29.8 ± 3.2**	NI[a]	0.48 ± 0.04
Isocorydine	30.55 ± 2.05**	NI[a]	NI[a]	1.90 ± 0.57*
Quercetin	0.44 ± 0.22	36.0 ± 4.0**	60.0 ± 7.0**	NI[a]
Quinic acid	12.3 ± 0.4**	NI[a]	NI[a]	NI[a]
Amikacin[b]	6.81 ± 0.132**	0.178 ± 0.002	0.029 ± 0.010	0.060 ± 0.009
Vancomycin[b]	0.075 ± 0.00	0.083 ± 0.011	0.035 ± 0.008	16.78 ± 1.662**

a) NI: No inhibition; b) Positive control; c) IC_{50}: 50% inhibitory concentration; d) SD: standard deviation; *** P < 0.001, ** P < 0.01 and * P < 0.05: statistically significant difference to the positive control; P > 0.05 no statistically significant difference to the positive control. One-way ANOVA followed by Dunnets t-test against vancomycin (*P. aeruginosa*, *S. aureus* and *M. smegmatis*) and amikacin (*E. coli*), n=3.

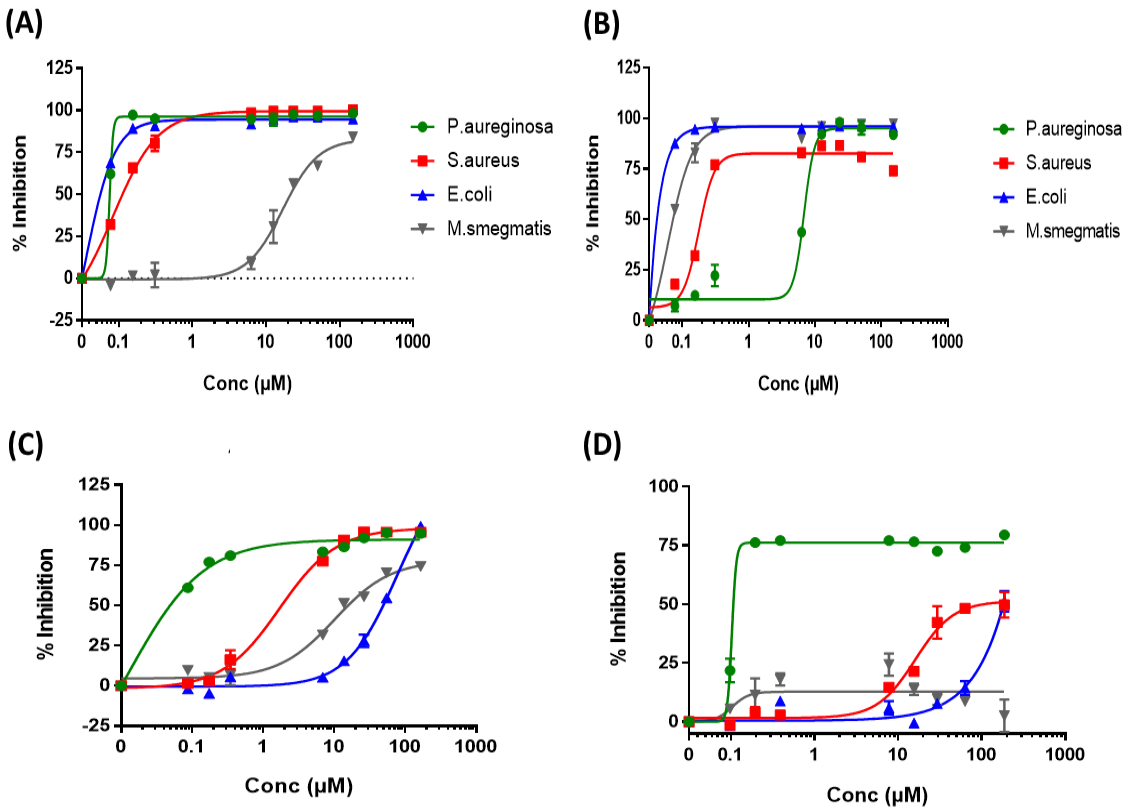

Figure 4. 2. Antibacterial growth assays. (A) In the presence of vancomycin. (B) In the presence of amikacin. (C) In the presence of adriamycin. (D) In the presence of annonacin.

4.5 Conclusion

Twenty-six compounds were putatively identified from the methanolic extract of the ripe fruit of A. triloba. Of the 26 compounds, 16 compounds had a statistically significant total antioxidant capacity compared to the positive control, Trolox. The antioxidant activity of these compounds is likely due to the compounds being phenolic compounds, known for their high antioxidant activity. High cytotoxicity was observed for adriamycin and annonacin against leukemia (HL-60), melanoma (SKMEL5), breast cancer (MCF-7), ovarian cancer (SKOV3), and prostate cancer (PC3) cell lines. These

results support the literature on adriamycin and annonaceous acetogenins for their antitumor activity. Twelve compounds displayed significant antibacterial activity against P. aeruginosa, S. aureus, M. smegmatis and E. coli, which supports the traditional use of the plant to treat skin and gastrointestinal tract infections. Among the 26 compounds analyzed for their bioactivity, adriamycin and quercetin demonstrated statistically significant activity in all the investigated bioassays. Additionally, except for the ARE activation studies, (-)-catechin gallate and (+)-syringaresinol displayed significant activity in all bioassays. The four lead compounds identified in the present study exhibited significant activity in one or more cancer cell lines and displayed antibacterial activity against one or more bacteria. The results in the present study validate the traditional usage of A. triloba as a potentially valuable resource for treating various diseases. These results highlight the importance of further research to explore the potential of A. triloba and its compounds in targeting antibiotic resistance in bacteria, inflammation, and investigating their anticancer potential.

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