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Evaluation of the Total Antioxidant Capacity of Three Selected Plants in Guyana

Bibi Ally-Charles [a*], Ede Tyrell [a], Basil Dey [b], Karishma Jeeboo [a], Andrew Hutson [a], Obena Vanlewin [a], Charlan Abrams [a] and Jamain Hatton [a]

[a] College of Medical Sciences, University of Guyana, Georgetown, Guyana.
[b] Faculty of Natural Sciences, University of Guyana, Georgetown, Guyana.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed to evaluate the leaf extracts of three commonly used medicinal plants in Guyana, namely *Brassica juncea* (mustard), *Struchium sparganophora* (antsbush), and *Psidium guajava* (guava), for their total antioxidant capacity.
Study Design: Experimental-based study.
Place and Duration of Study: The experiments were performed at the chemistry laboratory in the Faculty of Natural Sciences and the main laboratory in the College of Medical Sciences at the University of Guyana from September 2022 to August 2023.

**Corresponding author: Email: bibi.ally-charles@uog.edu.gy;*

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Methodology: The leaves of these plants were collected, washed, and air-dried at 28^0C for approximately one month, then pulverised in a sterilised food processor. Leaf extracts were then obtained by maceration with various solvents such as hexane, ethyl acetate, chloroform, and methanol; followed by filtration and rotary evaporation. The extracts obtained were then subjected to total antioxidant quantification via the phosphomolybdate method using a spectrophotometer. The total antioxidant capacity of the extracts was expressed as ascorbic acid equivalents.
Results: Antioxidant activity was seen for all the extracts but in varying quantities. The highest antioxidant capacity was observed for the *P. guajava* extracts with a mean of 5.2 mg/mL. The least activity was observed for the *S. sparganophora* extracts, with a mean of 0.9 mg/mL. The ethanolic extracts showed the highest antioxidant capacity (10.6 mg/mL for *P. guajava*; 2.5 mg/mL for *B. juncea*). The hexane extracts had the least (0.33 mg/mL for *P. guajava*; 0.24 mg/mL for *B. juncea*).
Conclusion: The leaves of *B. juncea*, *S. sparganophora,* and *P. guajava* have antioxidant capacity but those of *P. guajava* have greater potential for use as complementary and alternative treatments for chronic diseases.

Keywords: Antioxidants; phytochemicals; plant extracts; phosphomolybdate; ascorbic acid; chronic diseases.

1. INTRODUCTION

The association of free radicals in many diseases has been well studied. Free radicals are chemical species possessing an unpaired electron in the valence shell. They are highly reactive and unstable molecules. Free radicals are mostly oxygenated or nitrogenated and include superoxide, peroxide, nitric oxide, and nitrogen dioxide [1]. They are produced during metabolism and, when produced in excess, impair the important biological molecules in cells, thereby disrupting normal cell functions [2]. Free radicals may be the etiology of many chronic conditions including, cancer, diabetes, cardiovascular and degenerative diseases [3].

Antioxidants are compounds that can capture free radicals, thereby opposing oxidation. They function as free radical scavengers, and reducing agents [4]. They can reduce the oxidative damage associated with many diseases, and are therefore crucial in disease prevention and management [5]. Over the last few decades, the interest in plants as natural sources of antioxidants has increased [6].

Phytochemicals are bioactive compounds produced by plants for their protection [7]. Extracts prepared from various plants contain a variety of phytochemicals such as alkaloids, flavonoids, phenolics, and steroids [8]. These phytochemicals have several medicinal properties such as antimicrobial, anticancer, antidiabetic and antioxidant [9]. Phytochemicals like carotenoids, vitamins, and polyphenols may possess antioxidant and free radical scavenging properties [10]. Many investigations have been focusing on the phytochemical analysis of plants by extracting, isolating, and identifying bioactive compounds that have medicinal value.

Many plants have been studied for their various properties, such as antimicrobial properties. However, the literature reveals limited information about the antioxidant capacity of Guyanese indigenous plants such as *P. guajava*, *B. juncea*, and *S. sparganophora*.

B. juncea is known as mustard greens, and belongs to the Brassicaceae family [11]. It is a source of a variety of micronutrients, antioxidants, β-carotenoids and vitamins. *B. juncea* is used to treat a variety of non-communicable illnesses [12]. Phytochemical screening revealed the presence of flavonoids, tannins, saponins, glycosides, steroids/triterpenoids, anthraquinones, and polyphenols [13]. Phytochemical analysis revealed the presence of brassinosteroids such as castasterone, teasterone, typhasterol, and 24-epibrassinolide [14]. *B. juncea* leaves have antioxidant compounds that lower lipid peroxidation, oxygenation, and hyperglycemia [11]. However, it is unclear whether the species in Guyana have substantial amounts of antioxidants.

S. sparganophora is known as antsbush in Guyana and water bitter leaf in Africa. It belongs to the Asteraceae family [15]. It is used to treat a variety of communicable and non-communicable conditions, such as malaria, fungal infections, cancer, diabetes, and gonorrhea [16,17]. People in rural areas of Guyana use it to treat diaper rash and yeast infections [18]; while people in Nigeria uses it to treat malaria and gonorrhea [19]. Phytochemicals such as alkaloids, cardiac

glycosides, flavonoids, and hydrolyzable tannins were found in *S. sparganophora* [20]. *S. sparganophora* has antioxidants that scavenge free radicals, and inhibit α - amylase and glucosidase activity [21]. However, it is unclear whether the species in Guyana have a considerable quantity of antioxidants.

P. guajava is known as guava and belongs to the *Myrtaceae* family [22]. It has medicinal value worldwide. The fruit contains a high concentration of vitamin C and is used traditionally to control high blood pressure in some parts of the world [23]. The leaves possess phytochemicals like flavonoids, tannins, phenols, terpenoids, and glycosides [24]. Several phenolics such as catechins, gallic acid, chlorogenic acid, caffeic acid, epicatechin, rutin, quercetin, kaempferol, and luteolin have been identified in *P. guajava*; and these are associated with antioxidant activity [25]. However, it is unclear whether the species in Guyana have desirable antioxidant capacity.

The majority of the world's population (64%) utilises plants that contain phytochemicals as a substitute for conventional medications to treat chronic conditions [26]. They inhibit biochemical reactions, scavenge toxic chemicals, and improve the absorption of essential nutrients, among others [27]. Phytochemicals are not considered essential nutrients. Therefore, they are not consumed as food.

The extraction of phytochemicals from plants involves a series of steps. Techniques such as maceration, soxhlet, percolation, and decoction are used [9]. However, maceration is the most common. It involves soaking dried ground particles with solvents for several hours under occasional shaking [28]. Solvents that are polar, non-polar, and semi-polar are frequently used to extract a wide range of phytochemicals [29,30]. Studies have shown that methanol, ethanol, ethyl acetate, acetone, and hexane are some of the best solvents used [7]. Antioxidant activities can be investigated using assays like 1, 1-diphenyl-1-picrylhydrazil (DPPH), ferric-reducing antioxidant power (FRAP), 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and phosphomolybdate [31].

The DPPH assay assesses the ability of a substance to scavenge free radicals. It uses commercially available DPPH reagents [32]. The antioxidants in the substance would reduce the DPPH reagent to phenyl picrylhydrazine. The rate of this reaction depends on the ability of the substance to donate electrons [33]. When the substance accepts an electron, it becomes a stable compound in methanol and generates a maximum absorption value at 517 nm [34].

The FRAP assay is associated with the reducing potential of iron. Reductones donate electrons, permitting the reduction of ferric to ferrous irons. The quantity of ferrous complex formed is determined by Perl's Prussian blue formation at 700 nm. Iron is a reactive metal that can produce free radicals from peroxides by Fenton's reaction, and has been implicated in cardiovascular and other diseases. A reduction of ferrous levels in the Fenton reaction hinders oxidative stress [35].

The phosphomolybdate method assesses total antioxidant capacity (TAC). It was developed by Prieto and his colleagues in 1999. In this assay, Molybdenum (VI) is reduced to Molybdenum (V) by the substance in the sample. A green phosphate Molybdenum (V) complex is formed at an acidic pH [36]. This assay has been maximized to reflect linearity and reproducibility for evaluating the concentration of antioxidants in plants, and vitamin E in seeds and beans.

Studies suggest that phenolic compounds and flavonoids are useful antioxidants [37,38]. Evaluation of total phenolic content (TPC) and total flavonoid content (TFC) is useful in antioxidant studies. TPC can be evaluated via the Folin-Ciocalteu method, while the TFC can be evaluated by the aluminum chloride colourimetric method. The antioxidant capacity of phenolic compounds is mostly associated with their redox potential [32]. Phenolics are considered powerful free radicals scavengers and lipid peroxidation inhibitors. Studies have shown the association of TPC of plant extracts to their antioxidant activity [35,39]. Phytochemicals such as phenols and flavonoids in plant extracts can be screened for according to standard testing procedures [40].

In Guyana, the antioxidant properties of *P. guajava*, *B. juncea*, and *S. sparganophora* have never been explored. A review of the literature shows only a few studies that investigated the antioxidant activity of these plant leaves; however, the phosphomolybdate method was rarely explored. This novel study provides valuable insights into the antioxidant capacity of these plant leaves, and their potential to be used as complementary and alternative medicines for

treating chronic conditions. The objective was to measure the total antioxidant capacity of the leaves of these three plants. We specifically sought to find out which of the three plant leaf extracts has the highest antioxidant capacity and which solvent used in the extraction process is associated with the most antioxidant activity.

2. METHODS

This was an experimental-observational study carried out at the chemistry laboratory in Natural Sciences and the main laboratory in the College of Medical Sciences at the University of Guyana. Three medicinal plants in Guyana were evaluated in this study. They were *B. juncea, S. sparganophora*, and *P. guajava* (Fig. 1). The collection and preparation of the plant leaf extracts were performed from 2022 to 2023.

2.1 Collection of Plant Materials

Authentic *B. juncea* seeds were purchased from a certified botanist in Region 6 and were grown to obtain healthy mustard leaves. *P. guajava* leaves were collected from villages in Regions Three and Four. *S. sparganophora* leaves were collected from the National Agricultural Research & Extension Institute (NAREI) in Region 4 and a site in Kimbia, Berbice River (Region 6). The plants' leaves were identified by the Centre for Study of Biological Diversity, University of Guyana. Leaves that showed no sign of deterioration were used.

2.2 Preparation of Plant Leaf Extracts

The leaf extracts were prepared based on a method described by Tyrell and team in 2023 [41]. The leaves were washed thoroughly with water and then air-dried at 28^{o}C for 2-4 weeks. A sterilised food mixer was used to pulverise the dried leaves. The pulverised *B. juncea* leaves were macerated using three solvents, namely ethanol, ethyl acetate, and hexane, in separate jars. The pulverised *S. sparganophora* leaves were macerated using two solvents, namely ethyl acetate, and chloroform, in separate jars. The pulverised *P. guajava* leaves were macerated separately using four solvents, namely 95% ethanol, methanol, ethyl acetate, and hexane, in separate jars. A variety of solvents with different polarities were selected in the hope of extracting a wide range of compounds with antioxidant properties. The extracts were then filtered using a standard filtration apparatus and subsequently reduced under pressure at 45^{0}C using a RotaVap. The crude extracts were then ready for the next process.

2.3 Antioxidant Testing by the Phosphomolybdate Method

2.3.1 Preparation of reagents

The phosphomolybdate reagent was prepared by mixing equal volumes of 28 mM Sodium phosphate, 0.6 M Sulphuric acid, and 4.0 mM Ammonium molybdate. It was set aside in the dark until needed. This reagent was freshly prepared and used within two days.

2.3.2 Calibration curve of Ascorbic acid

A calibration curve of ascorbic acid was prepared by adding 0.1 mL aliquots of 0.0010, 0.0100, and 0.1000 mg/mL ascorbic acid solution to 0.1 mL of the freshly prepared phosphomolybdate reagent. The samples were mixed well and incubated at 95^{O}C for 90 minutes. After incubation, the absorbance for each concentration of ascorbic acid was taken at 695 nm against a reagent blank and recorded.

A-Mustard Leaves **B-Antsbush** **C-Guava leaves**

Fig. 1. Leaves of the plants investigated in this study

2.3.3 Processing of the leaf extracts

The leaf extracts were processed simultaneously. The crude extracts were taken as the 100 mg/mL concentration. An aliquot of 0.1 mL crude extract was added to 0.1 mL of the freshly prepared phosphomolybdenum. The samples were carefully mixed and then incubated at 95^{O}C for 90 minutes. After incubation, the absorbance for each extract was taken at 695 nm against a reagent blank and recorded. The TAC of the leaf extracts was estimated from the ascorbic acid calibration curve ranging from 0.05-5 mg/mL (R^2=0.9905) and expressed as ascorbic acid equivalents.

2.4 Phytochemical Screening

We identified the plant with the highest TAC and performed phytochemical screening for two essential antioxidants, phenols, and flavonoids, according to standard testing procedures [40].

2.4.1 Test for flavonoids

Screening for flavonoids was done by adding about 3 pieces of magnesium metal ribbon to 1 mL of the plant leaf extracts. Next, concentrated hydrochloric acid was added in a drop manner. A positive test was revealed by the formation of a pinkish-red colour [40].

2.4.2 Test for phenol

Screening for phenolic compounds was done by dissolving ferric chloride solution in 0.20g of the plant leaf extracts. A positive test was revealed by the formation of a green or dirty green precipitate [40].

3. RESULTS

Of the three plant leaf extracts evaluated, the most pronounced TAC was noted for the *P. guajava* leaf extracts (mean: 5.2 mg/mL) and the least was noted for *S. sparganophora* (mean: 0.9 mg/mL). The TAC of the various solvent fractions of the *B. juncea* leaves decreased in this order: hexane > ethyl acetate > ethanol. The TAC of the various solvent fractions of the *S. sparganophora* leaves decreased in this order: chloroform > ethyl acetate. The TAC of the various solvent fractions of the *P. guajava* leaves decreases in this order: hexane > methanol > ethyl acetate > ethanol. Antioxidant activity was seen for all plant leaf extracts but in varying quantities. The ethanolic extracts showed the highest TAC (10.6 mg/mL for *P. guajava*; 2.5 mg/mL for *B. juncea*). The lowest TAC was observed for the hexane extracts (0.24 mg/mL for *B. juncea*; 0.33 mg/mL for *P. guajava*) (Table 1).

Fig. 2 shows an ascorbic acid calibration curve with a correlation coefficient close to 1 (R^2=0.9999). This indicates a strong linear relationship between absorbance and concentrations and validates the readings obtained for the plant leaf extracts.

Table 2 shows that the ethanolic and methanolic extracts of the *P. guajava* leaves were positive for both flavonoids and phenols, while the ethyl acetate and hexane extracts were positive only for flavonoids (Table 2).

4. DISCUSSION

Antioxidants are compounds that prevent the oxidation of biological molecules by reducing the oxidising agents. They can destroy free radicals, thereby minimising oxidative stress associated with redox reactions. Essentially, they help prevent oxidative damage in humans and plants [5]. Antioxidant activity in plants is critical in protecting the human body from many deadly conditions, including cardiovascular diseases, cancer, and diabetes [41]. The antioxidant capacity of plants is solely related to the bioactive compounds present in them. The composition and concentration of these compounds in plants tend to vary from plant to plant [42]. Variations in bioactive compounds are also seen in different plant parts. In this current study, one sample, that is the leaves from three different plants indigenous to Guyana, was collected and investigated simultaneously using the phosphomolybdate method. This method evaluates the TAC regarding the reductive activity of phenolics and non-phenolic compounds.

Two to four different fractions of the crude extracts (100% concentrations) were used for comparison of their TAC. The *P. guajava* leaf extracts yielded the highest TAC, whereas the *S. sparganophora* gave the lowest. The polar ethanolic fractions gave the highest TAC, whilst the non-polar hexane extracts gave the least. Our study showed that polar solvents may be used to extract antioxidant compounds in higher amounts. Similar results were noted in other studies [7,29]. Solvents such as methanol and ethanol have high polarities and are used to

extract polar compounds. Examples of polar compounds include phenols and flavonoids, which are thought to be potent antioxidants [38]. A study showed that flavonoids are the most important phytochemical found in ethanolic *P. guajava* leaf extract and that flavonoids are associated with antioxidant activity [43,44]. Another study showed that polyphenols increase antioxidant activity [31]. Our study showed that flavonoids and phenols were present in both the ethanolic and methanolic *P. guajava* extracts. A methanolic crude extract was only investigated for *P.guajava* leaves. Surprisingly, this particular fraction did not yield a high TAC value as the ethanolic fraction. However, since we quantified TAC and screened for phenols and flavonoids; but did not quantify specific antioxidants, including phenolics and flavonoids, the claims made by previous studies may still stand [37,38]. We postulate that the ethanolic fraction had large quantities of phenols, and flavonoids along with other antioxidants.

Table 1. Total antioxidant capacity of the plant leaf extracts

Plant leaves	Type of extracts	TAC (mg/mL)
Brassica juncea	Hexane	0.24
	Ethanol	2.50
	Ethyl acetate	1.33
	Mean	1.36
Struchium sparganophora	Ethyl acetate	0.92
	Chloroform	0.51
	Mean	0.90
Psidium guajava	Ethyl acetate	5.84
	Ethanol	10.6
	Methanol	4.19
	Hexane	0.33
	Mean	5.24

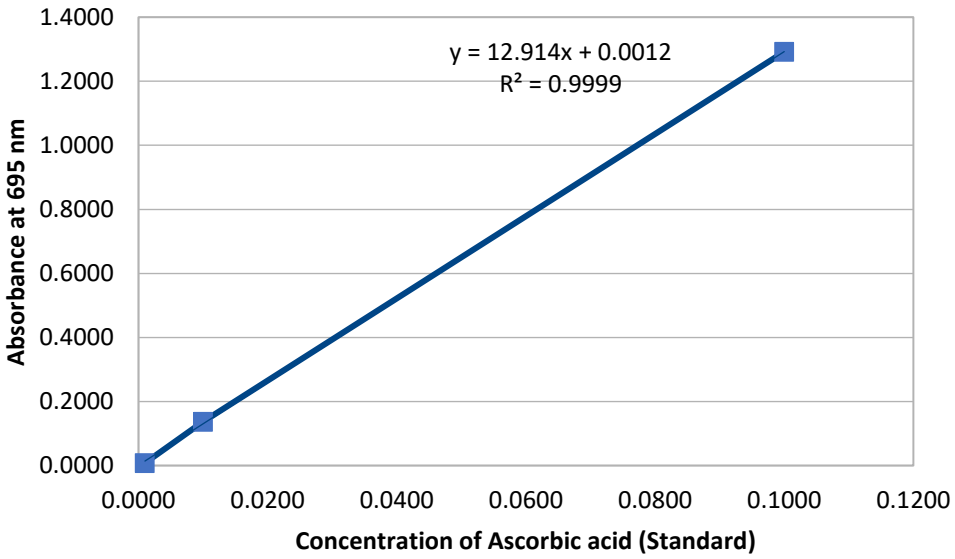

Fig. 2. Calibration curve for ascorbic acid

Table 2. Flavonoids and phenols screening of *P. guajava* leaf extracts

Phytochemicals	Flavonoids	Phenols
Ethanol extract	+	+
Methanolic extract	+	+
Ethyl acetate extract	+	-
Hexane extract	+	-

+ Presence, - Absence

4.1 *B. juncea* Leaf Extracts

Our study evaluated the TAC of *B. juncea* leaf extracts using three solvents (ethanol, ethyl acetate, and hexane) via the phosphomolybdenum assay. The crude extracts exhibited TAC ranging from 0.2- 2.5 mg/mL. Of the three solvents used, the most effective one was the polar solvent ethanol, followed by the semi-polar solvent ethyl acetate. The least effective solvent was the non-polar hexane. A similar finding was observed in another study, where the polar methanolic extract was most effective, followed by the semi-polar ethyl acetate extracts. The least effective solvent was the non-polar chloroform extract [45]. However, the DPPH method was employed. We could not find any study in the literature that examined the TAC of *B. juncea* leaf extracts via the phosphomolybdenum method. However, another study evaluated the antioxidant capacity of *B. juncea* seed extracts using the DPPH and ABTS assays using 30% ethanolic and water extracts. They found that the antioxidant activity using the DPPH assay with IC50 value ranged from 0.170 to 0.390 mg/mL and the ABTS assay with inhibition percent value ranged from 69 to 76% [46]. The results from our study were much higher when compared to the DPPH values obtained, however, it must be noted that the DPPH method tests for radical scavenging activity only while the phosphomolybdenum method considers the TAC.

4.2 *S. sparganophora* Leaf Extracts

Our study investigated the TAC of *S. sparganophora* leaf extracts using different solvents, namely ethyl acetate and chloroform, via the phosphomolybdenum assay. The crude extracts exhibited TAC ranging from 0.5-0.1 mg/mL. Of the two solvents used, the most effective one was the semi-polar ethyl acetate. We could not find any study in the literature that examined the TAC of *S. sparganophora* leaf extracts via the phosphomolybdenum method. However, a study investigated the antioxidant properties of the ethanolic extract, and found that it had a strong antioxidant capacity as characterized by its high TPC (5.4 g/100 g), reducing power (2.50), and free radical scavenging ability (65.2%) [47]. In another study, *S. sparganophora* leaf extracts destroyed DPPH radicals in a dose-dependent pattern (0-1.25 mg/mL) [21]. The results from our study were much higher when compared to the DPPH values obtained, however, it must be noted that the DPPH method tests for radical scavenging activity only while the phosphomolybdenum assay measures the TAC of the extracts.

4.3 *P.guajava* Leaf Extracts

Our study investigated the TAC of *P.guajava* leaf extracts using different solvents, namely ethanol, methanol, ethyl acetate, and hexane, via the phosphomolybdenum assay, and the most effective solvent was found to be ethanol since the highest concentration of the TAC was noted for the ethanolic extract. The next effective solvent was ethyl acetate, followed by methanol. The least effective solvent seemed to be hexane. A similar investigation of *P.guajava* leaf extracts using different solvents, namely hexane, ethyl acetate, butanol, and methanol via the ABTS and FRAPS assays showed that the methanolic extracts possessed the highest antioxidant activity [48], which was different from our finding. We postulate that if the authors had investigated the ethanolic extract in their study, a similar result would have been found. They also showed that the next effective solvent was butanol and ethyl acetate fractions, respectively, and the hexane fraction showed the least antioxidant activity, which corroborates our findings. Another study showed that the ethanolic extract had high radical scavenging and TPC activities, but the authors investigated the *P. guajava* peels [49]. A similar study was performed via the phosphomolybdenum assay and revealed that the leaf extracts showed stronger TAC than the fruit extracts [44].

Although our study only quantified TAC, a study had identified the main phenolic compounds in *P. guajava* leaf extracts as catechins, gallic acid, chlorogenic acid, caffeic acid, epicatechin, rutin, quercetin, kaempferol, and luteolin [25]. Another study showed that ethanol *P. guajava* leaf extract showed the strongest antioxidant activity with the Trolex equivalent antioxidant capacity (TEAC) value of 4.91 ± 0.050 mM trolox equivalents/mg extract [48], which coincides with our results for *P. guajava* leaves, where an average value of 5.24 mg/mL was found. A study claimed that *P.guajava* leaf extracts comprise a valuable possible source of natural antioxidants [50]. Another study investigated the antioxidant activity of *P. guajava* leaf and fruit extracts using four methods, including the phosphomolybdate assay. They found that both the leaf and fruit extracts destroyed DPPH radicals, with IC50 of 74.77 and 843.84 µg/mL, respectively, and both extracts showed low chelating activity for ferrous

ions with IC50 of 147.07 and 2105.05 µg/mL respectively [44]. The FRAP results increased with increasing concentrations of the leaf extracts (50 to 200 µg/mL), which implied an increase in reducing ability, but the fruit extract did not show considerable reducing activity. The phosphomolybdate test showed that the extracts and standards show evidence of an increase in absorbance, suggesting an increased reduction of Mo (VI) to Mo (V) by the antioxidant compounds in the leaf extracts [44].

According to the World Health Organization, Cancer is a leading cause of death globally. It accounts for approximately 10 million deaths and about one in six deaths in 2020 [51]. Cancer is one of the most common causes of death in Guyana, as mentioned by the Health Minister [52]. Guyana is rated amongst the highest in the world for Cervical Cancer incidence and deaths [53], and Breast cancer remains the number one cancer in Guyana [54]. Cancer treatments such as chemotherapy and radiography are usually expensive and have many detrimental side effects [55]. In addition, Diabetes mellitus, a chronic metabolic disorder, is the 8th leading cause of death and disability combined in the world [56]. Approximately 460 million persons across every country and age category were living with this disorder in 2019. The age-standardized death rate due to this metabolic condition was estimated at 20.9 deaths per 100,000 population. The age-standardised death rate from Diabetes is high in Guyana with about 82.6 deaths per 100,000 population [57]. Many of the currently used antidiabetic therapies have serious side effects, which may lead to hypoglycemia and cardiovascular and metabolic alterations [58]. Furthermore, cardiovascular diseases (CVDs) are deemed as another leading cause of death worldwide. About 17.9 million people died from CVDs in 2019, accounting for 32% of all global fatalities [59]. CVD is the most common non-communicable disease in Guyana. It accounts for 526 deaths per 100,000 individuals per year [60]. In 2017, the primary causes of death in Guyana were Ischemic heart disease and stroke.

Cancer, Diabetes, and Cardiovascular diseases are prevalent and leading causes of death globally and regionally. The search for cheaper complementary and alternative medicines to supplement conventional therapy without much toxicity and effects is necessary. Cancer, Diabetes, and Cardiovascular diseases lead to oxidative stress, and therefore, antioxidants have great potential for both adjunct and alternative therapies.

5. CONCLUSION

Our study shows *B. juncea* leaves, *S. sparganophora* leaves, and *P. guajava* leaves can be potential sources of natural antioxidants. However, *P. guajava* leaves possess the highest concentration of antioxidants and would be the preferred choice for treating many diseases. It may reduce the oxidative damage associated with cancer, diabetes, cardiovascular diseases, and many other conditions. These plants are easily accessible, widely available, and cost-effective and can therefore be used to supplement conventional therapy in the form of dietary supplements, or herbal decoctions and concoctions. There is also potential for their use as alternative therapies. Further testing should be done to investigate the toxicity levels of these plant leaves and to determine if they would be safe to be used as adjuncts or alternative therapies for Cancer, Diabetes, and Cardiovascular diseases.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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