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Comparative Study of Antioxidant Level and Activity from Leaf Extracts of Annona muricata Linn Obtained from Different Locations

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ABSTRACT

Annona muricata Linn possesses an anti-tumorigenic effect towards cancer. Several of its bioactive components have already been assessed in previous findings. However, none of the previous studies actually addressed the important consideration of the association between cultivation area of this medicinal plant and its bioactive compounds/antioxidants. In this study, the antioxidant level and antioxidant activity of 19 *Annona muricata* collected from different locations were evaluated by phenolic and flavonoid assays together with Oxygen Radical Absorbance Capacity (ORAC), Ferric Reducing Ability of Plasma (FRAP) and 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assays. M1 was found to have an attractive antioxidant profile as it had the highest content of phenolics (73.2 µg/mL GAE) and flavonoids (191.4 µg/mL CE) and also the highest antioxidant capacity in ORAC assay (254.7 µM). Additionally, it had a favourably high ferric ion reducing capacity (15.55 µM Fe2+/µg) and the best free DPPH-radical scavenging activity (IC_{50}=143.5 µg/mL). On the contrary, R1 showed the lowest level of phenolics with a GAE value of 21.92 µg/mL, ranked second lowest in flavonoid content (65.42 µg/mL CE), and it had the least antioxidant capacity in ORAC (94.66 µM), FRAP (4.17 µM Fe2+/µg) and DPPH assays (1597 µg/mL), making it the least desirable antioxidant source. Based on this finding, it was concluded that *Annona muricata* Linn had varied antioxidant levels and activity regarding its cultivation area; hence, it would be a guide in the selection of potential candidates for natural antioxidants in phytopharmacy.

Keywords: *Annona muricata*, antioxidant, phenolic, flavonoid, ORAC, DPPH, FRAP

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INTRODUCTION

Antioxidants are known for their role in stabilising or deactivating free radicals from attacking targets in biological cells (Sreeramulu et al., 2013). Free radicals like reactive oxygen species (ROS) and other oxidants induce damage to DNA and tissues, which results in the occurrence of various diseases including cancer (Franco et al., 2008). The most abundant antioxidants found in plants are polyphenolic compounds, which are the secondary plant metabolites that arise from a common intermediate, phenylalanine, or its close precursor, shikimic acid (Pandey & Rizvi, 2009). Polyphenolic compounds can be classified into four main classes, which include flavonoids, phenolic acids, stilbenes and lignans (Pandey & Rizvi, 2009). A previous study showed that the antioxidant activity of plant materials was correlated with the content of their phenolic constituents, suggesting that a high phenolic content indicates high antioxidant activity (Moein & Moein, 2010).

Annona muricata Linn, which belongs to the Annonaceae family, is commonly known as soursop, graviola or guanabana. It is native to sub-Saharan countries (Gavamukulya et al., 2014) though it is now widely cultivated in many tropical countries in the world such as India, Malaysia and Nigeria. Often, this plant is sought for its therapeutic effects. Each part of the tree i.e. the root, stem-bark, leaves, fruit and even the seed is used in traditional medicines around the world (Onyechi et al., 2012). The supposed therapeutic benefits of the soursop has attracted intensive research on the chemical composition of the leaves and seeds that has led to the finding of acetogenin compounds (Moghadamtousi et al., 2015). This molecular structure is a very potent compound against cancer as it deprives the high-energy demanding cancer cells of adenosine triphosphate (ATP) supply via the disruption of the mitochondrial electron transport system, resulting in apoptosis (Degli et al., 1994; McLaughlin, 2008)). These isolated compounds, which are secondary metabolites/antioxidants, answer the potential of the soursop for possessing anti-cancer, insecticidal, sedating as well as pain and immunosuppressing properties (Bermejo et al., 2005).

In the past, several studies focussed on antioxidant activity of extracts from pulps, leaves and peel of *Annona muricata* Linn. Akomolafe and Ajayi (2015) referred to a comparative study on antioxidant properties of the peel and pulp of ripe *Annona muricata* Linn that reported that the antioxidant potential in soursop peel was found to be significantly higher than in the pulp, as determined by ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Fe^{2+} chelation and hydroxyl scavenging tests. Furthermore, flavonoid and phenolic content was also higher in the soursop peel compared to the soursop pulp extract. Interestingly, *Annona muricata* possessed more potent in vitro antioxidant activity compared to the leaves of *Annona reticulata* and *Annona*

squamosa as revealed by Baskar et al. (2007). Additionally, *Annona muricata* Linn leaf aqueous extract was found to alleviate the pancreatic B-cells of Streptozotocin-treated diabetic rats by directly quenching lipid peroxides and indirectly enhancing production of endogenous antioxidants, thus addressing its antioxidant potential (Adewole & Caxton-Martins, 2006). Omale and Olakunle (2015) reported that the hepatoprotective and antioxidant activity of soursop stem bark extract against oxidative stress in rats induced by carbon tetrachloride (CCl_4) as determined from serum enzyme markers. According to Moghadamtousi et al. (2014), ethanol-induced gastric injury in rats could be treated by ethyl acetate extract of *Annona muricata* Linn leaves, which provide a suppressive effect against oxidative damage and a preservative effect on gastric wall mucus. Despite extensive research into the antioxidant level and activity possessed by *Annona muricata* Linn and its effectiveness in treating disease, a comparative study of the antioxidant level and activity of *Annona muricata* Linn obtained from different locations has not been reported. Nonetheless, previous studies have shown that there are different levels of antioxidant/phenolic content among plants of similar species (Lim & Quah, 2006). Therefore, the present study was initiated to determine the antioxidant level and antioxidant activity of aqueous leaf extracts of the *Annona muricata* Linn in different cultivation areas in Peninsular Malaysia.

MATERIALS AND METHODS

Plant Material and Preparation of Plant Extracts

Nineteen *Annona muricata* Linn leaf samples were collected from Peninsular Malaysia. The plant was identified and deposited with a voucher number by the Forestry Division, Forest Research Institute Malaysia (FRIM). The details of the sampling sites and voucher number of each sample are shown in Table 1. All of the 19 samples of old mature *Annona muricata* leaves were air-dried for a week before being ground to a powder using a grind mill. Later, about 10 g of each powdered sample was transferred into a Schott bottle containing 200 mL of sterile distilled water. The samples were incubated for three days with frequent agitation using an orbital shaker at room temperature. The mixture was then filtered to discard any solid material/marc. Finally, the filtrate extract was dried using the freeze dryer/lyophiliser machine to obtain the end product, *Annona muricata* crude extract (AMCE). The extract obtained was kept in sterile sample tubes and stored in a refrigerator at 4°C.

Determination of Total Phenolic Content in the Plant Extract

The total phenol content was determined using the Folin-Ciocalteau method developed by Singleton and Rossi (1965). A twofold serial dilution of positive standard (Gallic acid) was prepared from a 1 mg/mL concentration of the mother stock (i.e. 0.5 mg/mL; 0.25 mg/mL; 0.125 mg/mL;

Table 1
Sampling Sites of Annona muricata Linn in Peninsular Malaysia with the Code and Voucher Number of Each Sample

Location (State)	Code (Sampling Site)	Voucher Number
Johor	J1 (Muar)	ATCL1
Melaka	M1 (Jasin)	ATCL2
	M2 (Jasin)	ATCL3
	M3 (Bukit Beruang)	ATCL4
	M4 (Bukit Katil)	ATCL5
Negeri Sembilan	N1 (Kuala Klawang)	ATCL6
	N2 (Kuala Pilah)	ATCL7
	N3 (Kuala Pilah)	ATCL8
Selangor	B1 (Serdang)	ATCL9
	B2 (Serdang)	ATCL10
	B3 (Serdang)	ATCL11
Perak	A1 (Sungai Terap)	ATCL12
	A2 (Kg. Piandang)	ATCL13
	A3 (Kg. Batu Tujuh)	ATCL14
	A4 (Kg Bota)	ATCL15
Perlis	R1 (Kaki Bukit)	ATCL16
	R2 (Guar Jentik)	ATCL17
	R3 (Santan)	ATCL18
	R4 (Santan)	ATCL19

0.0625 mg/mL; 0.03125 mg/mL; 0.015625 mg/mL). The 19 extract samples were prepared at a concentration of 500 µg/mL. One millilitre of Gallic acid standard, extract samples and blank were transferred to separate test tubes. A total of 5 mL of diluted FC reagent was added into all the test tubes and incubated for 8 min at room temperature. Next, 4 mL of 7.5% sodium carbonate solution was added after some time and the solution was incubated for a further 2 h at room temperature before being read by a UV spectrophotometer at 765 nm wavelength.

Determination of Flavonoid Concentrations in the Plant Extract Samples

The flavonoid content was determined following the method described by Baba and Malik (2015). All extract samples were prepared at 1 mg/mL concentration. A serial dilution of standard (catechin) was prepared (i.e. 50µg/mL, 100µg/mL, 150µg/mL, 200µg/mL, 250µg/mL, 250µg/mL, 300µg/mL, 350µg/mL, 400µg/mL, 450µg/mL and 500µg/mL). A volume of 450µL of the extract samples and standard were added into different 1.5 mL tubes, after which 27µL of 5% $NaNO_2$ was added into each tube. The tubes were incubated for 6 min in a dark setting before the addition of 27µL of 10% AlCl3. An incubation period of 5 min was acquired in a dark setting. Afterwards, 180 µL of 1M NaOH was added into each tube followed by 216µL of dH_20. All the tubes were vortexed for 5 s before 300µL of the solution from each tube was transferred into a 96-well plate. The plate was read using ELISA at 430 nm wavelength.

Evaluation of Antioxidant Activity by ORAC Assay

All the samples were prepared at a concentration of 100ug/mL. Serial dilution of standard (Trolox) were prepared (i.e. 0 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM, 100 µM). A volume of 50 µL of the samples and Trolox was added in triplicate into a 96-well plate before a 50-µL of 60nM fluorescin was added into each well. The plate was incubated for 15 min at 37°C before 50 µL of

150mM AAPH solution was added into each well. The plate was read at 5-min intervals for 90 min (Exc=485nm and Emm=520 nm).

Evaluation of Antioxidant Activity by FRAP Assay

The FRAP assay was done according to Benzie and Strain (1996) with slight modification. The FRAP solution was prepared by mixing the 300-mM acetate buffer, 20-mM ferum trichloride and 10-mM TPTZ together at a ratio of 10:1:1. The FRAP solution was warmed at 37°C before use. All the extract samples were prepared at a concentration of 50 µg/mL. A volume of 50 µL of the extract samples and the positive control ($FeSO_4$) was added in triplicate into a 96-well plate. A volume of 250 µL FRAP solution was added into the wells containing the extract samples and positive control/ standard and incubated for 30 min in a dark setting. The plate was read with an ELISA plate reader at 593 nm wavelength.

Evaluation of Antioxidant Activity by DPPH Assay

DPPH-free radical scavenging activity was determined according to the method developed by Suja et al. (2005) with slight modification. An AMCE sample (50 µL), each with different concentrations (1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.63 µg/mL, 7.81 µg/mL) was added in triplicate in a 96-well plate. Then, a volume of 250 µL of 0.02 g/L DPPH solution in methanol was added into the wells containing the extract samples and allowed to stand for 30 min in a dark setting. The plate was read with an ELISA plate reader at 517 nm wavelength. The amount of AMCE sample needed to decrease the initial DPPH concentration by 50% was calculated graphically. Radical scavenging activity was calculated using the equation as follows:

$$\text{Scavenging activity} = \frac{[Abs_{control} - Abs_{sample}]}{[Abs_{control}]} \times 100$$

Statistical Analysis

All data were represented as means ±standard deviation of means. The analysis was performed using a one-way analysis of variance (ANOVA) and the group means were compared using the Duncan test. The statistical significance was evaluated at $p<0.05$ level.

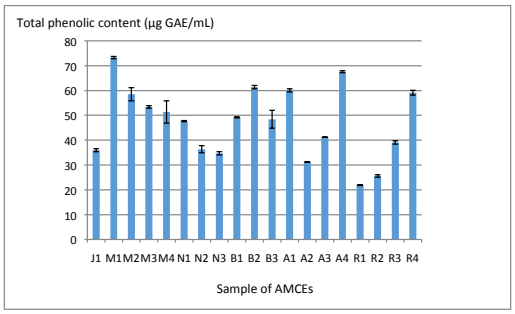

Figure 1. Total phenolic content for 19 samples of *Annona muricata* crude extract. The results obtained were equivalent to Gallic acid in µg/mL. Each value is expressed as the mean from three replicate measurements. The error bars indicate standard deviations

RESULTS AND DISCUSSION

The level of antioxidants in *Annona muricata* was determined by five antioxidant assays: total phenolic, total flavonoid, ORAC, FRAP and DPPH as depicted in Figures 1 to 5. M1 showed the richest source of phenolics (73.2 µg/mL GAE) followed by A4 (67.58 µg/mL GAE) and B2 (61.36 µg/mL GAE) samples, while R1, R2 and A2 possessed the lowest level of phenolics with

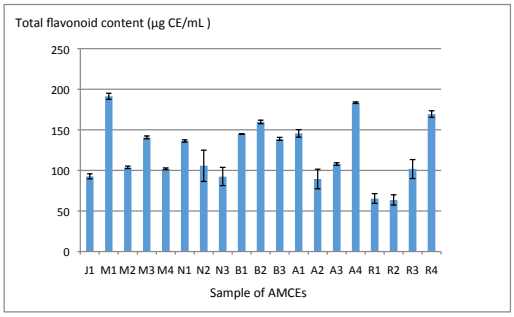

Figure 2. Total flavonoid content for 19 samples of *Annona muricata* crude extract. Antioxidant activity detected by flavonoid assay was represented as µg/mL catechin equivalent (CE). Each value is expressed as the mean from three replicate measurements. The error bars indicate standard deviations

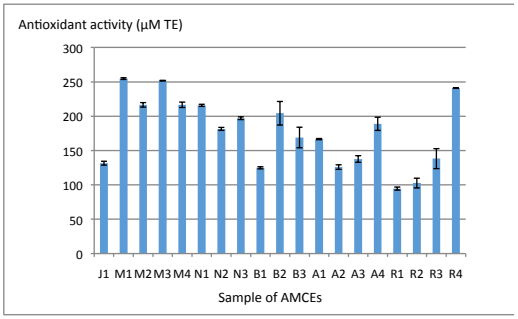

Figure 3. Oxygen radical absorbance capacity (ORAC) assay for 19 samples of *Annona muricata* crude extract equivalent to Trolox (µM). Each value is expressed as the mean from three replicate measurements. The error bars indicate standard deviations

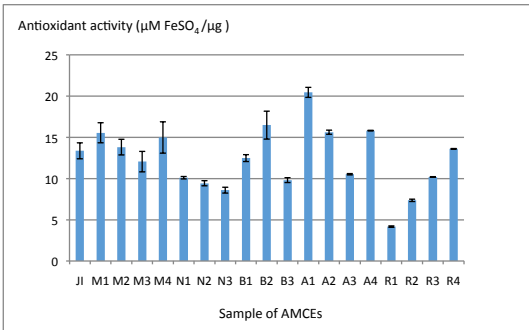

Figure 4. Ferric reducing ability of plasma assay (FRAP) for 19 samples of *Annona muricata* crude extract. Antioxidant activity detected by FRAP assay was represented as µM/µg FeSO4 equivalent. Antioxidant activity was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 µM/µg of FeSO4. Each value is expressed as the means from three replicate measurements and the error bars indicate standard deviations

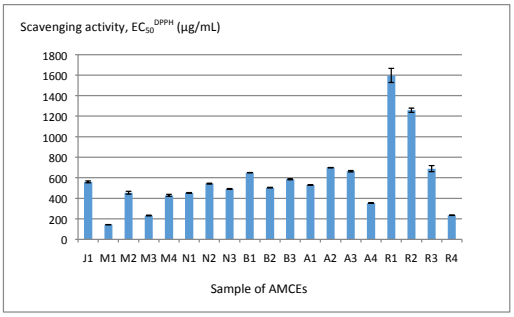

Figure 5. DPPH-free radical scavenging activity of 19 samples of *Annona muricata* crude extract. The scavenging activities of the AMCE samples were evaluated as the EC_{50}^{DPPH}, the concentration of antioxidants that reduces the DPPH-free radical by about 50%. Each value is expressed as the mean from the three replicates and the error bars indicate standard deviations

GAE value of 21.92 µg/mL, 25.56 µg/mL and 31.21 µg/mL, respectively (Figure 1). A similar order of the highest and lowest flavonoid content is recorded in Figure 2. M1 and A4 contained the highest level of the flavonoids (191.4 µg/mL and 183.5

µg/mL CE, respectively) while R2 (63.65 µg/mL CE) showed the lowest flavonoid level followed by R1 (65.42 µg/mL CE) (Figure 2). The radical scavenging capacity of AMCE was carried out by ORAC assay as depicted in Figure 3. The ORAC value varied from 94.66 to 254.7 µM Trolox equivalent per sample, which represented a variation of about threefold. The highest antioxidant capacity was found in M1 (254.7 µM), followed by M3 (251.7 µM). M2, M4 and N1 showed a similar level of ORAC values (216.5 µM, 216.6 µM and 215.8 µM, respectively). In this assay, R1 showed the lowest antioxidant potential (94.66 µM). The results of Ferric reducing capacity of AMCE are presented in Figure 4. The trend for the ferric ion reducing activities of the 19 AMCE samples tested varied approximately by fivefold. A1, B2, A4, A2 and M1 showed very strong ferric ion reducing activity (20.45, 16.48, 15.81, 15.62 and 15.55 µM Fe2+/µg, respectively). In this study, R1 and R2 extract possessed the lowest ferric reducing capacity (4.17 and 7.36 µM Fe2+/µg, respectively). The DPPH-scavenging activity of AMCE is shown in Figure 5. M1 showed the lowest EC50 value (143.5 µg/mL), indicating the highest antioxidant activity among the extract samples whereas R1 was the weakest antioxidant in reducing radical DPPH with EC_{50} value of 1597 µg/mL. In general, the DPPH-reducing power of the 19 AMCE samples varied approximately by elevenfold.

The comparative data of total phenolic and total flavonoid content presented in Figure 1 and 2, respectively, were determined primarily using the leaf aqueous extract from each *Annona muricata* Linn. sample. It is usual for antioxidant or secondary metabolite constituents to be distributed unevenly within each part of the plant and, to some extent, to not be expressed at all as it is mainly due to different morphology, anatomy and function of the plant organs (Mantle et al., 2000). However, the level of phenolic and flavonoid content varies throughout the soursop samples despite the fact that these samples were from the leaves of similar plant species. This anomaly could be related to the geographical/environmental differences that prevail where these soursop plants are cultivated (Gavamukulya et al., 2014; Scalzo et al., 2005). This is in agreement with the findings of Shams Ardekani et al. (2011), who found that pomegranates of different cultivars contained different levels of phenolics and flavonoids. The geographical difference of the cultivated plant means that each plant is exposed to a different climate and environmental stress factors such as humidity, temperature and soil composition (Gull et al. 2012). The synthesis and accumulation of secondary plant products are enhanced in stress environments such as a water-deficit condition (Selma & Kleinwachter, 2013). In a harsh environment, plants adjust their regulation of the phenylpropanoid biosynthesis pathway at multiple levels in response to the exogenous factors. For instance, the high intensity of visible light present would initiate the elevation of biosynthetic enzymes in the phenylpropanoid

pathway, which, consequently, enhances the production of anthocyanins and flavones as a means to reducing the amount of light available to the photosynthetic cells (Dixon & Paiva, 1995; Beggs et al., 1987). Moreover, flavonoids such as kaempferol derivatives are also highly induced to absorb UV rays to prevent damage that can result in cell death (Beggs et al., 1987). Previous studies have isolated and identified several polyphenol compounds from the leaves of the *Annona muricata* Linn. that include Gallic acid, epicatechine, catechine, quercetin, kaempferol, quercetin 3-O-rutinoside, quercetin 3-O-neohispredoside, quercetin 3-O-robinoside, kaempferol 3-O-rutinoside, quercetin 3-O-α-rhamnosyl-(1''→6'')-β-sophoroside and chlorogenic acid (Nawwar et al., 2012). The different levels of flavonoid and phenolic acid might be due to the level of expression of these aforementioned compounds present in the soursop samples.

Antioxidant activity of the soursop extract samples were evaluated by ORAC, FRAP and DPPH assay. At the present time, there is no single assay that is able to accurately reflect all of the radical sources or all antioxidants present in a certain food or extract sample (Dragovic-Uzelac et al., 2007; Prior et al., 2005). Therefore, it is best to analyse the antioxidant capacity of AMCE in several assays in order to generate a better antioxidant profile. The comparative data of the AMCE's antioxidant capacity in the ORAC, FRAP and DPPH assay are shown in Figures 3, 4 and 5, respectively, where the antioxidant activity of each sample varies in the assays. Only a few of the extract samples in ORAC assay had similar antioxidant capacity ranking order as those in the DPPH assay. This included M1, M3 and R4, which were observed to be the best three extract samples in scavenging the peroxyl radical and DPPH radical. The extract samples in the FRAP assay did not conform to the ranking order of both assays. M1, M3 and R4 were only ranked at 5th, 11th and 7th position, respectively, in reducing the Fe $(TPTZ)_2$ (III) in the FRAP assay. However, it is interesting that R1 and R2 were consistent in their ranking order, placed at the two lowest positions in all the assays. This event of ranking order discrepancy between assays in this present study is in accordance with the findings of Rufino et al. (2010). It is a plausible event considering the underlying reaction mechanisms of the assays employed, the source of oxidant/free radicals and method of quantitation that were different from each other (Huang et al., 2005). It is noteworthy that the three assays employed in this study were based on the capacity of the antioxidants possessed in the extract samples to break the radical chain sequences rather than inhibit the formation of the reactive oxygen species (chain-breaking antioxidants vs preventive antioxidants) (Ou et al., 2002). DPPH and FRAP assays, which are based on electron transfer, measure the antioxidant's capacity to reduce an oxidant that changes colour when reduced (Dudonne et al., 2009; Prior et al., 2005). ORAC assay, on the other hand, is based on the hydrogen atom transfer reaction, which involves the competition between antioxidant and

substrate for peroxyl radicals that are generated through the decomposition of azo compounds (Payne et al., 2013; Prior et al., 2005). DPPH and FRAP assay are simple, rapid and inexpensive but are subject to certain limitations. For instance, steric accessibility is a major concern in the DPPH assay (Prior et al., 2005) whereas in the FRAP assay, not all antioxidants possess the capability to reduce Fe(III) at a fast rate (or within a fixed time frame) as some polyphenols such as tannic acid, ferulic acid and quercetin might require a longer reaction time (Ou et al., 2002). To that extent, the 30-min reaction time in the FRAP assay is rendered insufficient for certain soursop extract samples; this affects the ranking order of the samples. On the other hand, an ORAC assay that uses the area under curve (AUC) approach for its kinetic reaction measurement allows the quantification of antioxidants that exhibit a distinct lag phase and those that have no lag phases, giving a more accurate result for antioxidant capacity (Huang et al., 2005). Above all, it is inappropriate to compare the antioxidant capacity ranking order of the extract samples between ORAC, FRAP and DPPH assays as they adopt different principles and none of the mentioned methods indicate the total antioxidant capacity of each extract. However, it is safe to assume that M1 could be considered the best antioxidant with regards to its high capacity (ranking order) across the assays.

CONCLUSION

This study has shown that Malaysia's *Annona muricata* Linn contains varied antioxidant levels and activity among the cultivars. Among the samples tested, M1 showed promising attributes to serve as an anti-oxidative agent due to possessing the highest phenolic and flavonoid content, as well as exhibiting the desirable antioxidant activity in the ORAC, FRAP and DPPH assays. On the other hand, R1 exhibited the lowest level of phenolic and flavonoid content; this explains their lowest rank in each of the antioxidant-activity assays. Based on the results presented, it is safe to make the assumption that *Annona muricata* Linn possesses different antioxidant levels and activity with regards to their cultivation areas. As it is anticipated that the soursop plant would be a useful pharmaceutical material in treating diseases associated with oxidative stress or cancer, the results provide a good platform for further evaluation and analysis of this plant. An extended study such as HPLC fingerprinting and other assays are needed to identify the specific compounds responsible for the varying antioxidant level and activity among the soursop samples.

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