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RESEARCH REPORT

Phytochemical content and antioxidant activity of different varieties of *Stevia rebaudiana*

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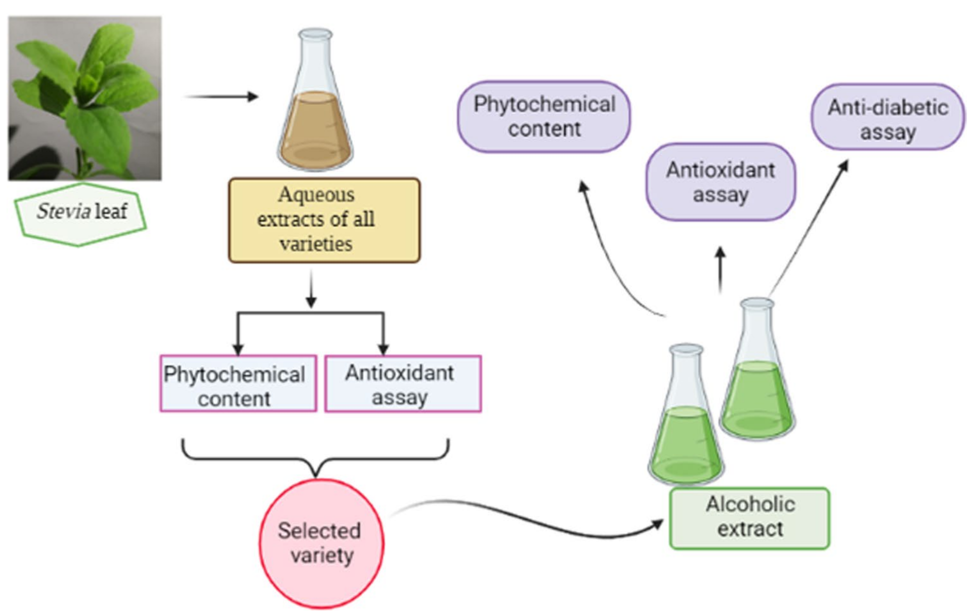
Abstract
The phytochemical content and antioxidant activity of leaves from five varieties of *Stevia rebaudiana* (Morita II, SA178, SA17, SA124, and Heam) were evaluated. Among the aqueous extracts of all varieties tested, the highest phytochemical content and antioxidant activity were both observed in the SA-178 variety. The values obtained from SA-178 for total phenolic, flavonoid, and FRAP content were 18.69 ± 0.014 mg of gallic acid equivalents per gram of dry weight, 3.91 ± 0.014 mg of quercetin equivalents per gram of dry weight, and 56.66 ± 0.01 mmol of Fe^{2+} per gram of dry weight, respectively. Extractions from this cultivar also showed the highest DPPH, ABTS, and Nitric oxide scavenging activity with IC_{50} values of 65.71 ± 0.56 μg mL^{-1}, 15.74 ± 0.27 μg mL^{-1}, 151 ± 0.03 μg mL^{-1}, respectively. For further analysis, alcohol extracts of SA-178 and Morita II (the most commonly used variety) were assessed for phytochemical content and antioxidant activity, and similar results were obtained. Aqueous and alcohol extracts of SA-178 were also studied for their antidiabetic properties, for which the aqueous extract showed the highest α-amylase and α-glucosidase activity with IC_{50} values of 1.15 ± 0.010 and 0.42 ± 0.01 mg mL^{-1}, respectively. As revealed by PCA analysis, a positive correlation was observed between phytochemical content and antioxidant activity. Therefore, SA-178 can be used as a sweetener in various products that will potentially also promote the management of oxidative-related diseases like diabetes.

Graphical abstract



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Keywords Antidiabetic activity · Antioxidant activity · Correlation · Different varieties · Phytochemicals · *Stevia rebaudiana*

1 Introduction

Stevia rebaudiana Bertoni tastes 200–300 times sweeter than sucrose and belongs to the Asteraceae family (Prakash et al. 2014). It is widely used as a flavoring ingredient for a variety of foods and beverages as well as a low-carbohydrate component in various diets (Elnaga et al. 2016). *Stevia* is known for its anti-obesity, antidiabetic, anti-hyperlipidemic, antioxidant, and anti-inflammatory effects (Ranjbar and Masoumi 2018). *Stevia* extract has been shown to decrease blood glucose levels and improve insulin resistance, as per Scaria et al. (2017). Synthetic antioxidants are less effective than *Stevia* against oxidative agents, and they may lead to other side effects as well such as skin discoloration, itching, bloating, flatulence, and diarrhoea, among others (Ruiz-Ruiz et al. 2015; DiNicolantonio et al. 2015; Wondafrash et al. 2020). Among natural antioxidants, phenolic acids play an important role. They are secondary metabolites formed from shikimic acid and pentose phosphate during the phenylpropanoid metabolization process in plants (Randhir et al. 2004). Antioxidants are substances that help prevent or reduce damage to cells affected by unstable molecules or free radicals (Pham-Huy et al. 2008). Sources of antioxidants may be natural or artificial, and some plant-based foods are considered especially rich in antioxidants (Brewer 2011). Natural antioxidants are generally the preferred alternative to manufactured antioxidants for defence against disease-causing free radicals (Nagmoti et al. 2012). Antioxidants also protect the body from other harmful molecules and reduce inflammatory reactions against allergens, toxins, and microbes (David et al. 2016). In this study, different varieties of *Stevia* were analyzed to determine which cultivar contains the highest quantity of plant-derived phytochemicals and highest antioxidant activity. Each plant species contains a different number of phytochemicals with variable antioxidant activities due to the presence of different enzymes in different plant lineages affecting secondary metabolites during their biosynthesis (Santos-Sánchez et al. 2019). Antidiabetic therapy attempts to establish normoglycemia and reduce insulin resistance in insulin-dependent (Type 1 Diabetes) and insulin-independent (Type 2 Diabetes) diabetic patients to enhance metabolic control and avoid future complications (Önal et al. 2005). Phenolic compounds, such as phenolic acids and flavonoids, covalently attach to alpha-amylase and change its activity by generating quinones or lactones that react with the nucleophilic groups of the enzyme molecule (Oyedemi et al. 2013). Polyphenols have also been shown to have various properties that block α-amylase and α-glucosidase, according to research. In this study, we evaluated the plant-based phytochemical content and antioxidant activity of various *Stevia* varieties.

2 Materials and methods

2.1 Plant materials

The different varieties of *Stevia,* i.e. Morita II, SA178, SA17, SA124, and Heam, were collected from Organic Innovation, Guwahati, Assam, and Jamuna Biotech farms in Pune, India. All plant varieties were identified as *Stevia rebaudiana* (Ref No. RC-14/2020-21) by taxonomist Dr. Keshava H Korse, Bhandimane Life Science Research Foundation, Karnataka. Plants were maintained in a greenhouse, and leaves were harvested from 3-month-old plants. The leaves were cleaned, air-dried at 28 ± 2 °C for 7–8 days, crushed into powder, and stored in an airtight container until use.

2.2 Chemicals

All chemicals and reagents used in this analysis were analytical grade. SRL Pvt. Ltd. (Mumbai) provided 2,4,6-tripyridyl-S-triazine (TPTZ), sodium nitroprusside (SNP), p-nitrophenyl glucopyranoside (pNPG), 2,4-dinitrophenylhydrazine (DNPH), naphthylethylenediamine dihydrochloride (NED), sulphanilamide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, gallic acid (GA), Trolox, quercetin (Q), curcumin, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), α-amylase, and α-glucosidase. Commercial acarbose (Glucobay®) was purchased from the market.

2.3 *Stevia* leaf extraction

The aqueous extract (AE) was made according to the procedure given by Woelwer-Rieck et al. (2010) with slight modifications. The dried leaf powder (3 g) was combined with 50 mL distilled water, vortexed for 1 h in a water bath at 100 °C, then centrifuged for 15 min at 4500 RPM. The filtrate was collected using Whatman no. 1 (11 µm pore size) filter paper and kept at 0–4 °C until use. The varieties with high phytochemical content and antioxidant activity were used for the alcohol extractions.

Methanol (MEs) and ethanol extracts (EEs) of dried leaves were obtained according to Al-Manhel and Niamah

(2015). The leaf powder (5 g) was combined with 50 mL of methanol or ethanol and kept for 24 h in a shaking incubator at 200 rpm. The supernatants were filtered using Whatman no. 1 filter paper and kept at 0–4 °C until needed.

2.4 Phytochemical content

2.4.1 Determination of total phenolic content

The phenolic compounds were determined using the Folin-Ciocalteu technique, which is based on phenolics reducing the phosphorwolframate-phosphomolybdate complex, with slight modification (Singleton and Rossi 1965). Absorbance was measured at 765 nm. Results were obtained by comparing the absorbance of each sample to a standard curve (0–250 mg mL^{-1} gallic acid). Three replicates of the experiment were carried out. The total quantity of phenolic compounds in the sample was estimated as mg of gallic acid equivalents (GAE) per gram of dry weight of the sample ($r = 0.99$).

2.4.2 Determination of total flavonoid content

The flavonoid content was determined by measuring the absorbance at 415 nm using a modified aluminum chloride method (Dewanto et al. 2002). The results were obtained by comparing each sample's absorbance to a standard graph (0–100 mg mL^{-1} of quercetin). Three replicates were used in the study. The total quantity of flavonoid components in a sample was measured in quercetin equivalents (QE) per gram of dry weight ($r = 0.99$).

2.5 Antioxidant assays

2.5.1 DPPH radical scavenging activity

All extracts were tested for their ability to scavenge DPPH radicals, according to Mitra and Uddin (2014). Thirty minutes of incubation in the dark at 27 ± 2 °C was performed on the samples. Absorbance at 517 nm was then measured against a methanol blank. Ascorbic acid was employed as a positive control. Percent inhibition may be calculated using this formula:

$$\%Inhibition = \frac{Absorbance(Blank - Test)}{Absorbance(Blank)} \times 100$$

The IC_{50} (µg mL^{-1}) of an antioxidant extract was also determined, which is the lowest inhibitory concentration necessary to quench 50% of the preliminary DPPH.

2.5.2 ABTS scavenging activity

The ABTS scavenging analysis of all extracts was performed according to Ayyash et al. (2018), with some changes. After adding 2.45 mM potassium persulphate to a 7 mM ABTS aqueous solution, the mixture was incubated for 16 h at 27 ± 2 °C in the dark. This mixture was incubated for an additional 30 min in the dark at 27 ± 2 °C after plant extracts at various doses (0–10 mg mL^{-1}) were added. As a control, we used ABTS and methanol instead of an extract to evaluate the absorbance at 734 nm. In this test, Trolox was utilized as a control. The formula used to determine the percentage of inhibition:

$$\%Inhibition = \frac{Absorbance(Blank - Test)}{Absorbance(Blank)} \times 100$$

The IC_{50} (µg mL^{-1}) of an antioxidant extract, which is the lowest inhibitory concentration necessary to quench 50% of initial ABTS, was also determined.

2.5.3 Ferric reducing antioxidant power (FRAP) assay

According to Chu et al. (2000), the FRAP test was conducted with all extracts. To fit within the linearity range, sample solutions were first diluted with deionized water to a specific concentration before being analyzed. 3 mL of FRAP reagent was preheated to 37 °C. The absorbance was measured at 593 nm after 4 min, with 100 µL of sample being added to the FRAP reagent along with 300 µL of deionized water. Values were calculated using the Fe^{2+} equivalent (FE) calibration curve and expressed in mM of Fe^{2+} equivalent (FE) per gram of dry weight of the sample. There was a linearity range of 0.1–1.0 mM on the calibration curve, and ascorbic acid was utilized as a reference.

2.5.4 Nitric oxide radicals scavenging activity

Nitric oxide in the SNP solution combines with oxygen to generate nitrite ions at physiological pH, which can be measured using the Griess-Ilosvay reaction (Mandal et al. 2011). Sulphanilamide was used to diazotize nitrogen ions, which were subsequently coupled with NED, and the pink color generated was measured spectrophotometrically at 540 nm and compared to the blank sample. Triplicates of each test were run and curcumin was used as standard. The formula to estimate the percentage of inhibition is as follows:

$$\%Inhibition = \frac{Absorbance(Blank - Test)}{Absorbance(Blank)} \times 100$$

The IC_{50} (μg mL^{-1}) of an antioxidant extract, which is the lowest inhibitory concentration necessary to quench 50% of initial nitric oxide, was also determined.

2.6 Antidiabetic assays

The variety with the highest phytochemical content and antioxidant activity was used in the following assays.

2.6.1 In vitro α-amylase inhibitory assay

Extracts were tested for their ability to inhibit α-amylase using a modified Ali et al. (2006) protocol. Absorbance was measured at 595 nm using commercial acarbose (Glucobay®) in the range of 0–2.5 mg mL^{-1}, and the inhibitory activity of α-amylase was calculated as follows:

$$\%\alpha\text{−}amylase\ Inhibition = \frac{Absorbance(Blank - Test)}{Absorbance(Blank)} \times 100$$

2.6.2 α-Glucosidase inhibitory assay

A study by Kim et al. (2000) investigated the impact of extracts on the activity of α-glucosidase. By measuring p-nitrophenol generated from pNPG at 405 nm and using commercial acarbose (Glucobay®) at concentrations of 0–10 μg mL^{-1} as a standard, α-glucosidase activity was determined. The following formula was used to determine the activity:

$$\%\alpha - glucosidase\ Inhibition = \frac{Absorbance(Blank - Test)}{Absorbance(Blank)} \times 100$$

2.7 Statistical analysis

For the analysis, Graph pad Prism 8 was utilized. The findings of each experiment were acquired from three separate experiments done in triplicate and were represented as mean ± SD. Tukey's Multiple Comparisons Tests was used to assess significance, and the findings were expressed as $p < 0.05^{*}$, $p < 0.01^{**}$, or $p < 0.001^{***}$. ANOVA was used to generate confidence intervals for all pairwise differences in factor level means while keeping the family error rate to a minimum. This approach modifies the confidence level for each interval to ensure that the resulting simultaneous confidence level equals the specified value. Principal component analysis (PCA) was used to perform multivariate analysis using MINITAB software version 20.3.0.0 for data analysis.

3 Results and discussion

3.1 Plant varieties

Five different varieties of *Stevia,* i.e. Morita II, SA178, SA17, SA124, and Heam, were used in this study (Fig. 1).

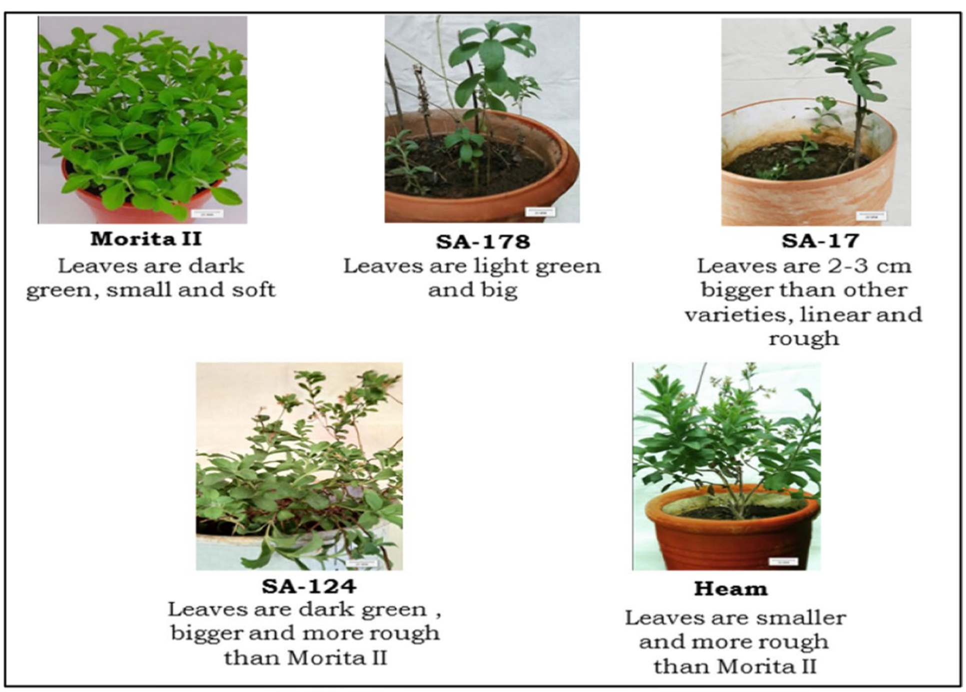

Fig. 1 Varieties of *Stevia* plants

3.2 Extraction yield

The effects of water and organic solvents (methanol and ethanol) on the extraction yield of *Stevia rebaudiana* were investigated. The results revealed a considerable variation in extraction yield when different solvents were used. Among the solvents studied, distilled water gave the highest extraction yield (80%), followed by methanol (75%), and ethanol (70.2%), showing that the strong polarity of water improves extraction efficiency.

3.3 Phytochemical

3.3.1 Total phenolic content

Phenolics are mainly associated with defence mechanisms in plants as they are essential in dealing with oxidative stress (Lin et al. 2016). Due to this property of phenolics, the total phytochemical content of all *Stevia* varieties was estimated. The total phenolic content of all varieties showed a significant level of $p<0.001$ when compared with the Morita II variety. The total phenolic content of all varieties was in the range of 4–19 mg GAE g^{-1} DW (Fig. 2). With a significance level of $p<0.001$, SA-178 had the highest phenolic content, at 18.69 ± 0.014 mg GAE g^{-1} DW, while SA-17 had the lowest phenolic content at 4.27 ± 0.010 mg GAE g^{-1} DW. A study by Yu et al. (2017) revealed that *Stevia* extract contains 20.85 mg GAE g^{-1} DW. *Stevia* AE was found to have 15.50 mg GAE g^{-1} DW of phenolics by Gaweł-Bęben et al. (2015), 25.6 mg GAE g^{-1} DW by Yildiz-Ozturk et al. (2015), and 28.40 mg GAE g^{-1} DW by Ruiz-Ruiz et al. (2015). According to Shukla et al. (2012), *Stevia* AE has 56.74 mg GAE g^{-1} DW of phenolic. This difference in values might be attributed to different plant varieties being used or environmental variables such as minerals present in the growing area and geographical location (Lopes et al. 2018). The alcohol extracts contained 5–11 mg GAE g^{-1} DW of total phenolic content (Fig. 2). SA-178 had the highest phenolic content among all alcohol extracts at 10.49 ± 0.044 mg GAE g^{-1} DW in an ME with a $p<0.001$ significance value. Meanwhile, EE of SA-178 contained only 5.91 ± 0.022 mg GAE g^{-1} DW, with $p<0.001$. This variation in values may be due to differences in the polarity of the compounds, which can explain changes in solvent efficiency (Ngo et al. 2017). Results from Garcia-Mier et al. (2021) and Yu et al. (2017) showed MEs of *Stevia* content led to 0.948 and 25.25 mg GAE g^{-1} DW of phenolics, respectively. Other studies reported EEs of *Stevia* contained 86.47 and 85.91 mg GAE g^{-1} DW of phenolics (Ciulu et al. 2017; Covarrubias-Cárdenas et al. 2018).

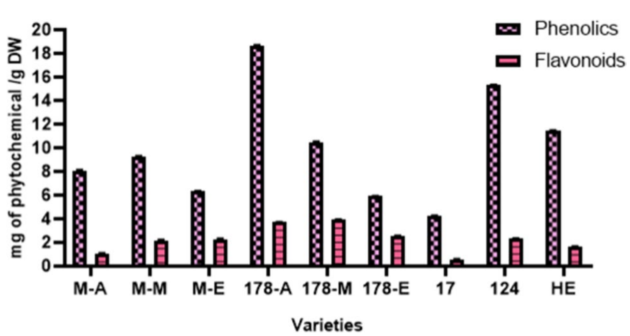

Fig. 2 Total phenolic and flavonoid content of *Stevia* extract. Results are reported as mean ± SD of triplicate tests, with the same significance levels (***$p<0.001$). (M-A-Morita II AE; M-M-Morita II ME; M-E-Morita II EE; 178-A-SA178 AE; 178-M-SA178 ME; 178-E-SA178 EE; 17-SA-17; 124-SA124; HE- Heam)

3.3.2 Total flavonoid content

Flavonoids play an important role in oxidative stress by regulating cellular activity and protecting against free radicals (Kumar and Pandey 2013). They also assist the human body in protecting itself from regular stress and toxins (Panche et al. 2016). As per Ruiz-Cruz et al. (2017), they are beneficial to the body because of their antioxidant, antidiabetic, and antiglycation properties and they protect the body from oxidative stress by acting as radical scavengers. Therefore, the total flavonoid content of all extracts was measured and 0.5–4 mg QE g^{-1} DW were detected with a significance level of $p<0.001$, as shown in Fig. 2. SA-178 had the highest flavonoid yield of 3.72 ± 0.014 mg QE g^{-1} DW, with a significance value of $p<0.001$ compared to the other samples. The AE of SA-17, on the other hand, had the lowest flavonoid concentration at 0.59 ± 0.010 mg QE g^{-1} DW, with a significance level of $p<0.001$. This disparity might be explained by plants developing in different environments, leading to varying primary and secondary metabolite synthesis and deposition (Marrassini et al. 2018). In an AE of *Stevia*, Gaweł-Bęben et al. (2015) and Lemus-Mondaca et al. (2018) reported 3.85 and 0.79 mg QE g^{-1} DW, respectively. Jahan et al. (2010) and Ruiz-Ruiz et al. (2015) reported 125.64 and 36.7 mg QE g^{-1} DW of flavonoids in an AE of *Stevia*, respectively. A significance level of $p<0.001$ was reported for all alcohol extracts, with flavonoid concentrations in the range of 2–4 mg QE g^{-1} of DW (Fig. 2). Accordingly, the ME of SA-178 had the highest flavonoid content of 3.91 ± 0.044 mg QE g^{-1} of DW ($p<0.001$), while the ME of Morita II had the lowest flavonoid content of 2.20 ± 0.036 mg QE g^{-1} of DW ($p<0.001$) (Fig. 2). Differences in the polarity of the compounds can explain the observed variation in the efficacy of solvents (Ngo et al. 2017). Garcia-Mier et al. (2021) and Atas et al. (2018) reported MEs of *Stevia* containing 0.165 ± 0.030 mg Rutin equivalents g^{-1} and 98 mg QE g^{-1} of DW of flavonoids, respectively. The EE of *Stevia* showed 125.64 and

10.91 mg QE g^{-1} DW of flavonoids in Jahan et al. 2010 and Zaidan et al. (2019), respectively.

3.4 Antioxidant assay

Antioxidants improve general health by helping to neutralize free radicals (Lobo et al. 2010) which are formed continuously in the human body. In the absence of antioxidants, free radicals are thought to cause significant damage very quickly, potentially leading to death (Sharma et al. 2012). As a result, our bodies must maintain a healthy equilibrium of free radicals and antioxidants (Lobo et al. 2010).

3.4.1 DPPH assay

DPPH can donate hydrogen molecules (Baumann 1979). As a result, it is a widely-accepted method for evaluating plant extract antioxidant activity. By adding the extract in a concentration-dependent manner, the DPPH solution is reduced to diphenyl picryl hydrazine, and the remaining DPPH content is determined. This technique has been widely utilized to predict antioxidant activity due to the small amount of time needed for analysis. In this investigation, the DPPH scavenging activity of the *Stevia* varieties was found to range from 65 to 95 µg mL^{-1}. SA-178 exhibited the highest DPPH activity and the lowest IC_{50} value of 65.71 ± 0.56 µg mL^{-1} with a significance level of $p < 0.001$ (Fig. 3). This might be because polyphenols and tocopherol can scavenge DPPH radicals by donating hydrogen (Rahman et al. 2015). The SA 178 variety showed a similar IC_{50} value to ascorbic acid, and therefore was not significant. SA-17, on the other hand, exhibited the lowest DPPH activity and the highest IC_{50} value of 94.87 ± 0.47 µg mL^{-1} with a significance value of $p < 0.001$. According to the findings, all *Stevia* extracts exhibited radical scavenging activity via electron transfer or hydrogen donation. Therefore, these extracts may be utilized as antioxidants that readily produce protons that can be used as free radical inhibitors. The IC_{50} values published by Kharchouf et al. (2017) and Rahim et al. (2016) were 0.56 and 38.87 mg mL^{-1}, respectively. Shukla et al. (2012) and Ruiz-Ruiz et al. (2015), on the other hand, reported IC_{50} values of 83.45 and 335.94 µg mL^{-1}, respectively. The alcohol extracts' DPPH scavenging activities were determined to be 11–71 µg mL^{-1} (Fig. 3). Among all extracts, the ME of SA-178 exhibited the lowest IC_{50} value of 10.84 ± 0.52 µg mL^{-1} with a significance level of $p < 0.001$. ME of Morita II possessed the highest IC_{50} value of 70.31 ± 0.47 µg mL^{-1} ($p < 0.001$) (Fig. 3b). Jahan et al. (2010) and Tavarini and Angelini (2013) observed IC_{50} values of 23.7 and 250 µg mL^{-1}, respectively, for ME. The IC_{50} value for ethanol extracts against DPPH was reported to be 93.46 µg mL^{-1} (Shukla et al. 2009) and 23.70 µg mL^{-1} (Jahan et al. 2010). These differences in results might be explained by the various extraction methods employed.

3.4.2 ABTS assay

Potassium permanganate or potassium persulphate are strong oxidizing agents that react with the ABTS salt to form ABTS. This approach is fast and may be utilized in both aqueous and organic solvent systems with a wide variety of pH values. It also offers a high degree of repeatability and is easy to implement, receiving significant attention as a result (Ratnavathi and Komala 2016). The ABTS technique is commonly used to measure antioxidant activity because ABTS free radicals become stable by absorbing a hydrogen ion from the antioxidant, resulting in a reduction in blue coloration (Lee et al. 2015). In comparison to Trolox, the

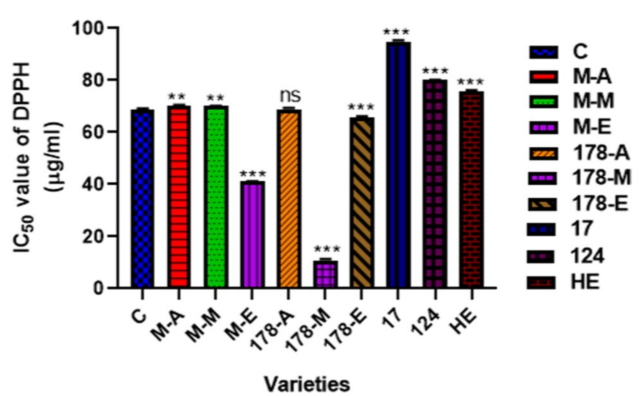

Fig. 3 DPPH activity of *Stevia* extract. Results are reported as mean ± SD of triplicate tests, with different significance levels (**$p < 0.01$, ***$p < 0.001$, ns: non-significant). (C-control; M-A-Morita II AE; M-M-Morita II ME; M-E-Morita II EE; 178-A-SA178 AE; 178-M-SA178 ME; 178-E-SA178 EE; 17-SA-17; 124-SA124; HE-Heam)

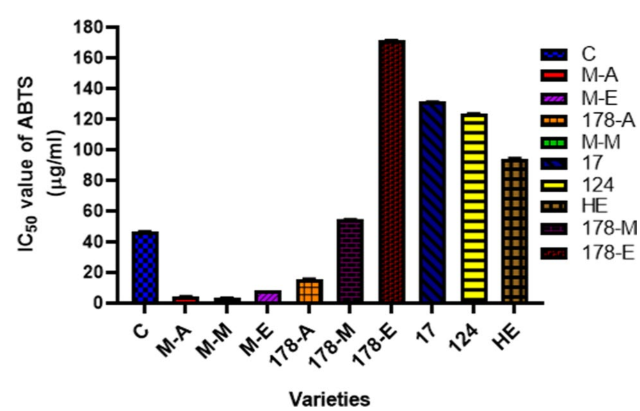

Fig. 4 ABTS activity of *Stevia* extract. Results are reported as mean ± SD of triplicate tests, with the same significance levels (***$p < 0.001$). (C-control; M-A-Morita II AE; M-M-Morita II ME; M-E-Morita II EE; 178-A-SA178 AE; 178-M-SA178 ME; 178-E-SA178 EE; 17-SA-17; 124-SA124; HE-Heam)

ABTS test assesses the antioxidant's capacity to recover ABTS produced in the aqueous phase. The ABTS scavenging activity of all varieties was found to be in the range of 4–132 μg mL^{-1}. Morita II exhibited the highest ABTS activity and the lowest IC_{50} value of 4.52 ± 0.07 μg mL^{-1}, with a significance level of $p < 0.001$ (Fig. 4). SA-178, on the other hand, had an IC_{50} of 15.74 ± 0.15 μg mL^{-1}, while SA-17 had the lowest activity, again with a significance level of $p < 0.001$. For the AE of *Stevia* against ABTS, Phansawan and Poungbangpho (2007) and Tadhani et al. (2007) found IC_{50} values of 1.67 and 38.24 μg mL^{-1}, respectively. ABTS scavenging activity of the alcohol extract was determined to be 3–172 μg mL^{-1}. Of all extracts, Morita II had the lowest IC_{50} at 3.62 ± 0.07 μg mL^{-1}, which was statistically significant ($p < 0.001$) (Fig. 4). The EE of SA-178, on the other hand, exhibited the highest IC_{50} value of 171.54 ± 0.15 μg mL^{-1}, with a significance level of $p < 0.001$. The synthesis and accumulation of different primary and secondary metabolites are affected by plant growth conditions, which could explain this variation in results (Labarrere et al. 2019). Phansawan and Poungbangpho (2007) reported an IC_{50} value of 2.85 ± 0.92 μg mL^{-1} for ME against ABTS. Gaweł-Bęben et al. (2015), on the other hand, reported an IC_{50} value of 1.34 μg mL^{-1} for an EE of *Stevia.*

3.4.3 FRAP assay

Reducers, which function as antioxidants by disrupting superoxide radical chains by donating electrons, are typically associated with the presence of reducing power (Mayakrishnan et al. 2013). In the FRAP assay, the Fe^{3+}/ferricyanide complex is reduced to Fe^{2+}/ferrous by reducers in the antioxidant sample. *Stevia* AE was tested for its ability to reduce the Fe^{3+} ferricyanide complex to the ferrous form by donating an electron. Reducing abilities varied from 13 to 57 mmol of Fe^{2+} g^{-1} of dry weight ($p < 0.001$) for the extracts. Among all varieties, the highest FRAP activity (56.66 ± 0.02 mmol of Fe^{2+} g^{-1} DW) was observed for AEs of SA-178 with a significance level of $p < 0.001$ (Fig. 5). Conversely, the lowest FRAP activity of 13.14 ± 0.07 mmol of Fe^{2+} g^{-1} DW was observed for the AE of SA-17 with a significance threshold of $p < 0.001$. Alvarez-Robles et al. (2016) reported the FRAP activity of 1.00 mmol of Fe^{2+} g^{-1} DW for an AE of *Stevia*. In contrast, Ortiz-Viedma et al. (2017) reported FRAP activity varying from 0.12 to 0.18 mmol Fe^{2+} g^{-1} DW in various extracts of *Stevia*. The FRAP activity of the alcohol extracts was found to be in the range of 14–36 mmol of Fe^{2+} g^{-1} DW. Among all extracts, the highest FRAP activity of 35.43 ± 0.24 mmol of Fe^{2+} g^{-1} DW was observed for the ME of SA-178 with a significance of $p < 0.001$ (Fig. 5). The lowest FRAP activity of 14.16 ± 0.02 mmol of Fe^{2+} g^{-1} DW was observed for the EE of SA-178 with a significance value of $p < 0.001$. Tavarini et al. (2013) showed that an ME of *Stevia* had a total antioxidant capacity of 0.813 mmol of Fe^{2+} g^{-1} DW. Lucho et al. (2018, 2019), reported 1350 and 48 μmol Fe^{2+} g^{-1} DW FRAP activity of the EE, respectively. In contrast, Ortiz-Viedma et al. (2017) reported FRAP activity varying from 0.12 to 0.18 mmol Fe^{2+} g^{-1} DW in various extracts of *Stevia*. These variations might be attributed to different *Stevia* varieties, harvest season, and solvent extraction methods used in their studies (Silva et al. 2018).

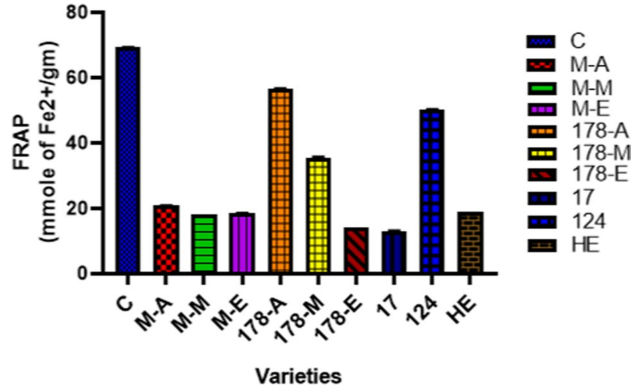

Fig. 5 FRAP activity of *Stevia* extract. Results are reported as mean ± SD of triplicate tests, with the same significance levels (***$p < 0.001$). (C-control; M-A-Morita II AE; M-M-Morita II ME; M-E-Morita II EE; 178-A-SA178 AE; 178-M-SA178 ME; 178-E-SA178 EE; 17-SA-17; 124-SA124; HE-Heam)

3.4.4 RNS assay

Sodium nitroprusside in an aqueous pH solution creates nitric oxide, which then interacts with oxygen to yield nitrite ions, which may then be detected using the Griess reagent, according to the method in Boora et al. (2014). Because of their redox capabilities, phenolics can operate as reductants, simple hydrogen donors, and oxygen quenchers, as well as potential metal chelators (Boora et al. 2014). Using in vitro nitric oxide radical quenching, antioxidant activity may be determined (Nagmoti et al. 2012). Scavengers of nitric oxide compete with oxygen, resulting in a reduction in nitrite ion production (Ebrahimzadeh et al. 2010). Nitric oxide is readily scavenged by flavonoids (Lakhanpal and Rai 2007). In its aerobic form, nitric oxide is a highly unstable species that interact with oxygen to create the stable products nitrate and nitrite via the intermediates NO_2, N_2O_4, and N_3O_4 (Patel et al. 2010). The extract's nitric oxide scavenging activity was determined to be between 151–390 μg mL^{-1}. Among all extracts, the maximum activity with the lowest IC_{50} value of 151 ± 0.028 μg mL^{-1} was observed for SA-178, which was still higher than curcumin (55.87 ± 0.054 μg mL^{-1}), with a significance of $p < 0.001$ (Fig. 6). Morita II was found to

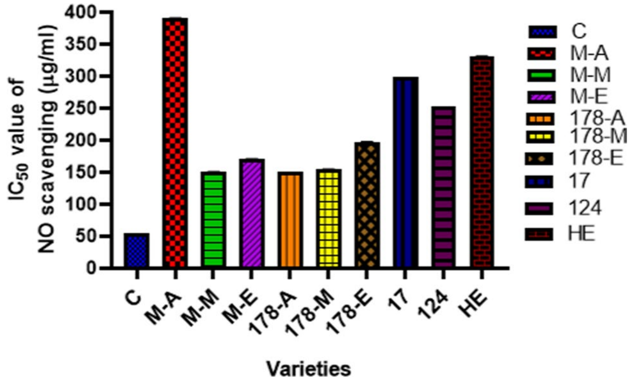

Fig. 6 Nitric oxide scavenging activity of *Stevia* extract. Results are reported as mean ± SD of triplicate tests, with the same significance levels (***$p < 0.001$). (C-control; M-A-Morita II AE; M-M-Morita II ME; M-E-Morita II EE; 178-A-SA178 AE; 178-M-SA178 ME; 178-E-SA178 EE; 17-SA-17; 124-SA124; HE-Heam)

have the highest IC_{50} value, with a significance threshold of $p < 0.001$. Shukla et al. (2012) found that *Stevia* AE had a nitric oxide scavenging activity of 98.73 µg mL^{-1}. The alcohol extract's nitric oxide scavenging activity ranged from 150 to 197 µg mL^{-1}. The ME of Morita II had the greatest activity and the lowest IC_{50} value at 150 ± 0.04 µg mL^{-1} among all alcohol extracts, with a significance of $p < 0.001$ (Fig. 6b). The EE of SA-178 had the lowest activity and the highest IC_{50} value of 197 ± 0.04 µg mL^{-1}, with a significance threshold of $p < 0.001$. Shukla et al. (2009) found that *Stevia* EE has a nitric oxide scavenging efficiency of 132.05 µg mL^{-1}. Although these effects are modest, they are notable because secondary metabolites are responsible for reacting to environmental changes, suppressing protein synthesis, and regulating enzyme activity, but can also lead to cell death (Ozcan and Ogun 2015; Marrassini et al. 2018).

Among the varieties analyzed in this study, Morita II and SA178 showed the highest phytochemical content and antioxidant activities in the AE, so they were used for further studies using different solvent systems like methanol and ethanol.

3.5 In vitro α-amylase and α-glucosidase inhibitory assays

In managing type 2 diabetes, Krentz and Bailey (2005) recommended blocking the enzymes α-amylase and α-glucosidase to prolong carbohydrate digestion, which leads to low postprandial glucose levels and reduces the impact one's diet on hyperglycemia (Bischoff 1994). When α-glucosidase is inhibited, carbohydrate digestion is limited and blood sugar levels are lowered (Van de Laar et al. 2006). Acarbose and miglitol are two α-glucosidase inhibitors that prevent carbohydrates from being absorbed in the gut. Several studies have shown that these inhibitors are effective in preventing or postponing

Table 1 Inhibition of α-amylase and α-glucosidase activity of *Stevia* extracts

Extracts	IC_{50} value	
	α-amylase (mg mL^{-1})	α-glucosidase (mg mL^{-1})
Acarbose	0.25 ± 0.035	0.49 ± 0.020
AE	1.15 ± 0.010***	0.42 ± 0.01**
ME	1.23 ± 0.02***	0.54 ± 0.03*
EE	1.70 ± 0.02***	0.56 ± 0.01**

Data presented as mean ± SD (n = 3)

a decrease in glucose tolerance in diabetics. Because plant phenols may partially block α-amylase, they can be utilized as therapeutic agents to treat secondary complications of diabetes (Chethan et al. 2008). According to Rasouli et al. (2017), the binding affinity of most phenolic compounds is higher for α-amylase than α-glucosidase, which has higher docking energy and reduces the inhibitory effect. As a result, polyphenols' primary structure can affect their inhibitory action on α-amylase and α-glucosidase activity (Zaidan et al. 2019). It has been shown by Kazi (2014) that plant-based phenolic compounds can inhibit the digestive enzymes α-amylase and α-glucosidase, lowering blood sugar levels and making them effective antidiabetic medications. Inhibition of α-amylase and α-glucosidase activity by *Stevia* was investigated using AE, ME, and EE of the SA178 variety as it showed higher phytochemical content and antioxidant activity than the other varieties tested. The AE showed the highest α-amylase and α-glucosidase inhibitory activity. In the AE, α-amylase, and α-glucosidase showed the lowest IC_{50} value of 1.15 ± 0.010 ($p < 0.001$) and 0.42 ± 0.01 mg mL^{-1} ($p < 0.01$), which was higher than the values for acarbose of 0.25 ± 0.01 and 0.49 ± 0.01 mg mL^{-1}, respectively (Table 1). The ME and EE showed 1.23 ± 0.02 and 1.70 ± 0.02 mg mL^{-1} of α-amylase and 0.54 ± 0.03 and 0.56 ± 0.01 mg mL^{-1} of α-glucosidase activity, respectively. Ruiz-Ruiz et al. (2015) reported the IC_{50} values of 200 µg mL^{-1} for the α-amylase activity of the Morita II variety. Recent research by Zaidan et al. (2019) found that *Stevia* leaf extracts had an IC_{50} value of 13.73 µg mL^{-1} for α-amylase activity. Compared to other extracts, AEs exhibited the highest activity, which may be linked to the presence of steviol glycosides (Rasouli et al. 2017). This can be utilized for the management of diabetic complications (Ruiz-Ruiz et al. 2015).

3.6 Statistical analysis

3.6.1 Correlation between phytochemicals and antioxidants

Phenolic and flavonoid compounds are essential antioxidants that deactivate free radicals by donating hydrogen atoms. As reported in previous research and the present study, polyphenols are present in AEs, MEs, and EEs. Studies on *Ipomoea aquatica, Rosa damascene, Foeniculum vulgare, Stachys lavandulifolia, Stevia rebaudiana*, and *Salvia hydrangea* have revealed that total phenol and flavonoid content and antioxidant capacity are linearly related (Shukla et al. 2009; Safari et al. 2018; Aryal et al. 2019; Ali et al. 2021). In this study, the AEs of Morita II, SA-17, SA-124, the ME of Morita II, and EE of SA-178 had the greatest correlation between DPPH and ABTS (Table 2) and between DPPH and RNS. The ME of SA-178 showed the highest correlation of 0.995. In the case of FRAP, however, a strong correlation between DPPH and ABTS was observed in SA-124 and Heam (Table 3). Rajurkar and Hande (2011) observed a strong relationship between ABTS and FRAP levels for herbal medicines using a similar technique. Leaf extracts with high amounts of phenolics and flavonoids may have significant levels of antioxidant activity (Khiraoui et al. 2017). Aryal et al. (2019) observed substantial associations between antioxidant capacity and total phenols (DPPH, $R^2 = 0.75$; H_2O_2, $R^2 = 0.71$) and total flavonoids (DPPH, $R^2 = 0.84$; H_2O_2, $R^2 = 0.66$) at a 95% confidence interval.

3.6.2 Principal component analysis

Principal component analysis (PCA) reduces the complexity of high-dimensional data while preserving trends and patterns. PCA geometrically projects data onto smaller dimensions known as principal components (PCs) in order to obtain the best statistical summary using a limited number of PCs (Jolliffe and Cadima 2016). PCA was used to examine the multidimensional properties of five different *Stevia* plant varieties. It accomplishes this by reducing the data to fewer dimensions, which serve as feature summaries. High-dimensional data are particularly prevalent in biology and develop when several characteristics, such as the activity of many enzymes, are assessed for each *Stevia* variety. The PCA findings were used to create the projection plot (Fig. 7), which shows the similarity of *Stevia* leaf extracts from different varieties. PCA should be used primarily for highly linked variables. To minimize data dimensionality and extract the signal, a simple scatterplot may be used to view the data and discover clusters if two major components concentrate more than 80% of the total variance (Lever et al. 2017). In this study, IC_{50} values from the DPPH, ABTS, Nitric oxide scavenging analysis, FRAP, total phenolic, and

Table 2 Relations between TPC and DPPH, RNS and FRA, and DPPH with ABTS

Extract	TPC (R^2)				DPPH and ABTS(R^2)	DPPH and FRAP(R^2)
	DPPH	ABTS	Nitric oxide	FRAP		
Morita II-AE	0.961	0.990	0.916	0.980	0.984	0.908
Morita II-ME	0.950	0.969	0.759	0.960	0.963	0.927
Morita II-EE	0.766	0.967	0.911	0.869	0.609	0.833
SA 178-AE	0.719	0.992	0.863	0.970	0.822	0.715
SA 178-ME	0.525	0.911	0.459	0.998	0.529	0.781
SA 178-EE	0.910	0.913	0.499	0.966	0.967	0.718
SA 17-AE	0.985	0.957	0.975	0.957	0.962	0.958
SA 124-AE	0.879	0.887	0.610	0.947	0.928	0.996
Heam-AE	0.612	0.994	0.849	0.995	0.658	0.666

Data presented as mean ± SD (n = 3)

Table 3 Relations between TFC and DPPH, ABTS, RNS, and FRAP

Extract	TPC (R^2)				ABTS and FRAP (R^2)	DPPH and RNS(R^2)
	DPPH	ABTS	Nitric oxide	FRAP		
Morita II-AE	0.947	0.992	0.931	0.986	0.980	0.795
Morita II-ME	0.936	0.967	0.757	0.955	0.993	0.788
Morita II-EE	0.766	0.957	0.912	0.887	0.765	0.949
SA 178-AE	0.707	0.992	0.858	0.976	0.974	0.963
SA 178-ME	0.515	0.893	0.448	0.993	0.907	0.995
SA 178-EE	0.897	0.915	0.489	0.961	0.845	0.764
SA 17-AE	0.968	0.960	0.982	0.960	0.975	0.966
SA 124-AE	0.879	0.886	0.609	0.946	0.927	0.880
Heam-AE	0.604	0.993	0.860	0.994	0.999	0.785

Data presented as mean ± SD (n = 3)

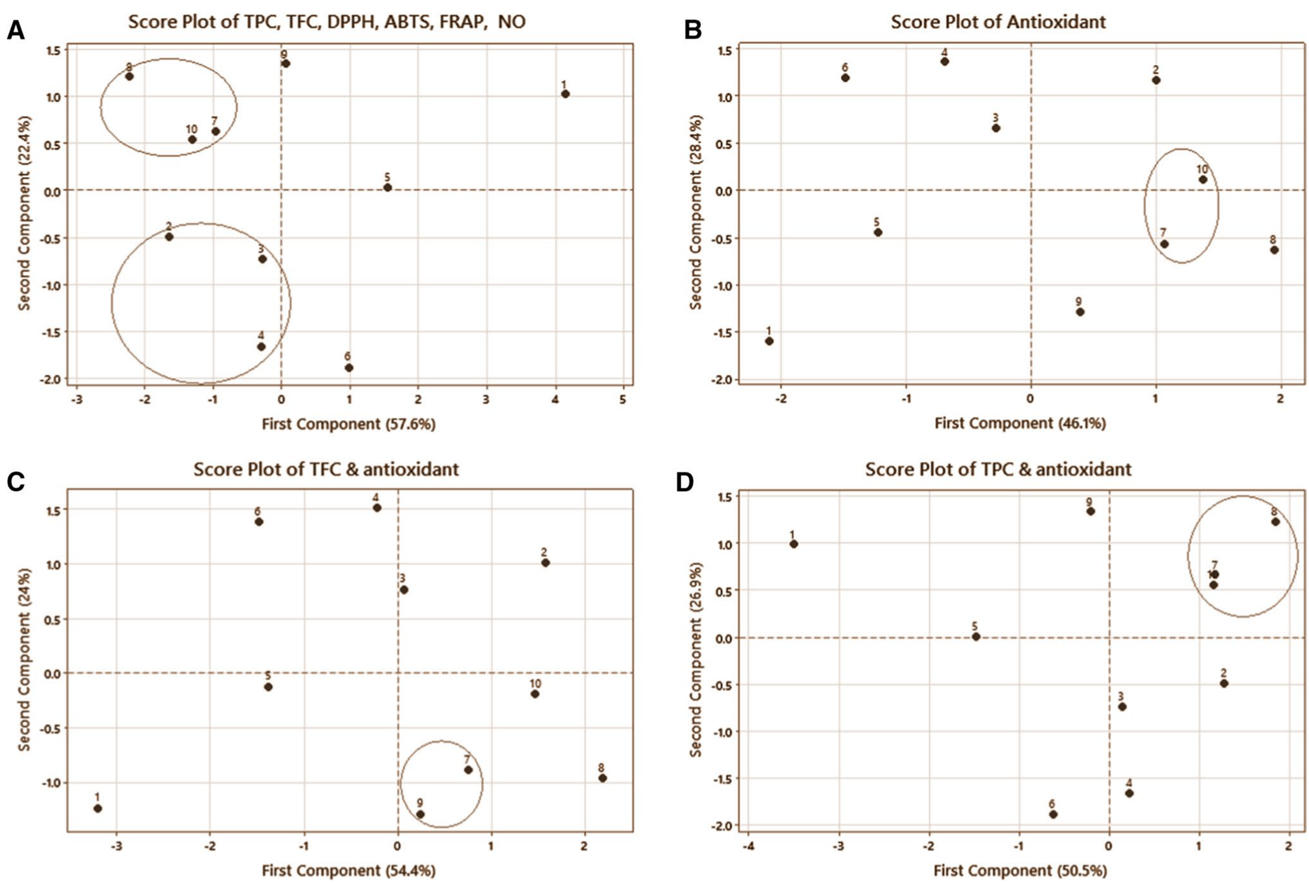

Fig. 7 Principal component analysis (PCA). **a** TPC, TFC, and antioxidants. **b** Antioxidant. **c** TPC and antioxidant. **d** TFC and antioxidant

total flavonoid contents were used to generate the loading plot of *Stevia*. IC_{50} values from DPPH, ABTS, Nitric oxide scavenging assays, FRAP, total phenolic, and total flavonoid contents of *Stevia* samples were shown in Fig. 7a. All samples were discovered to be scattered in an unorganized manner. PCA does not function effectively for data reduction if the association between variables is weak. However, by showing considerable similarities, some samples were classified into two clusters, one is of aqueous, methanol, and ethanol leaf extracts of the Morita II variety, and another is of EE from SA-178 with AE of 17 and Heam. The EE of SA-178 and Heam remained closer to each other in the PCA plot when the samples were grouped by all antioxidant tests, as shown in Fig. 7b. As shown in Fig. 7c, when the samples were categorized by total flavonoid content and antioxidants, an EE of SA-178 and an AE of SA-124 formed a cluster. In contrast, when the samples were categorized by total phenolic content and antioxidants, as shown in Fig. 7d, an EE of SA-178 and an AE of SA-17 and SA-124 formed a cluster. The EE of SA-178 appeared in all clusters in all figures. Total phenolic content has a strong relationship with antioxidant activity (Garcia-Mier et al. 2021). The presence of phenolic compounds such as flavonoids (Pérez et al. 2014) and stevioside in *Stevia* leaves contributes to its antioxidant capacity (Tavarini et al. 2020). Even though total flavonoid concentration in *Stevia* is higher than total phenolic acids, total flavonoid content was less strongly linked to antioxidant activity (Barroso et al. 2018).

4 Conclusion

The present study aimed to determine which type of *Stevia* has the highest phytochemical content and antioxidant properties. These active compounds in medicinal plants help treat diseases. Molecules derived from natural sources can be considered for use in the development of safer antidiabetic medicines for long-term usage. The extract was shown to have relatively high amounts of total phenolics and flavonoids, both of which are important in preventing free radical oxidation. According to our findings, *Stevia* includes virtually all types of phytochemical components and has antioxidant activity at varying doses. In this study, the AE of the SA-178 variety had a high phytochemical content and antioxidant activity. This result is also correlated with PCA analysis. The antioxidant capacity of the extracted fraction may be useful in avoiding or delaying the progression of different oxidative stresses. The antioxidant activities of secondary metabolites in plants might explain their therapeutic properties. As a result, the antidiabetic effect of this variety was investigated further. The AE exhibited notable activity in this study, suggesting that it might be a promising option for advanced antidiabetic medicines. Because the plant has a high concentration of these bioactive chemicals, it is likely to have a wide range of therapeutic properties, including antioxidant and antidiabetic properties. The findings of this study show that *Stevia* AE might be employed as a potential natural antioxidant source.

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Author contributions The study's inception and design were done by PJ. RJ planned the experimental setup. RS conducted the experiment, collected data, and analyzed it. SP ensured that the plants in the greenhouse are properly maintained and edited the manuscript. MB did the statistical analysis and edited the manuscript. KT provided the resources and edited the manuscript. RS also wrote the first draft of the manuscript and other authors provided feedback. The final manuscript was reviewed and approved by all authors.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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