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Research Article

Antioxidant Marvels : Tapping into *Tinospora crispa* and *Tinospora cordifolia* bioactive potentials via Antioxidant, Antiglycation and GC-MS Analysis

ABSTRACT

Tinospora crispa and *Tinospora cordifolia* are plant species that are commonly used in traditional medicine, such as Ayurvedic medicine, renowned for their therapeutic roles in addressing diverse health issues, including diabetes. These plants are esteemed for their ability to counter oxidative stress through electron donation which is a prominent feature of antioxidant. However, a sole assessment of their antioxidant effectiveness is insufficient to holistically understand their antioxidative capabilities. The primary objective of this study was to study the antioxidative and antiglycation properties exhibited by *T. crispa* and *T. cordifolia*. This evaluation encompassed a range of tests measuring radical scavenging activity (DPPH assay), capacity for reducing ferric ions (FRAP assay), and their antiglycation potential (BSA-MGO assay). To further enhance this investigation, GC-MS analysis was employed to identify compounds with antioxidative properties within *T. crispa* and *T. cordifolia*. The stems and leaves of *T. crispa* and *T. cordifolia* underwent solvent extraction using 90% methanol and hot distilled water. Notably, the methanolic extract of *T. cordifolia* displayed the most robust radical scavenging activity, evident from its lowest IC_{50} value, 0.03 ± 0.00 mg/mL in the DPPH assay. Conversely, the methanolic extract of *T. crispa* exhibited the least IC_{50} value, 0.19 ± 0.00 mg/mL in the FRAP assay. Additionally, the methanolic extract of *T. cordifolia* showcased a minimal IC_{50} value 0.52 ± 0.18 mg/mL in the BSA-MGO antiglycation assay. It's worth noting that the methanolic extracts of both *T. crispa* and *T. cordifolia* outperformed their hot water counterparts in terms of antioxidative activity, potentially due to the presence of phytochemical compounds such as phenol, 4-vinyl guaiacol, guaiacol, syringol, and vanillin in the methanolic extracts. The study highlights the potent antioxidative properties of *T. crispa* and *T. cordifolia* in supporting their traditional medicinal use and leads the way for the development of antioxidant therapies, particularly for managing oxidative stress-related conditions such as diabetes.

Key words: Antiglycation, FRAP, scavenging, *T. cordifolia*, *T. crispa*.

INTRODUCTION

In most parts of the world, herbal products have been used extensively as part of the practice of complementary and alternative medicine (CAM). *T. crispa*, a climbing shrub, is a member of the Menispermaceae botanical family which can be found in Asian and African rainforests. The foliage, stem, and roots of *T. crispa* have been traditionally utilized as alternative remedy for diverse medical conditions. (Ahmad *et al.*, 2016 ; Thomas *et al.*, 2016). Another Menispermaceae family member known as *T. cordifolia* is used in Ayurvedic medicine to address a multitude of disorders including diabetes mellitus (Kumar 2015). A review by Singa *et al.*, (2022) revealed that both *T. crispa* and *T. cordifolia* stems and leaves are high in phytochemical content such as phenols that are widely recognised for their antioxidant and/or hypoglycemic properties. In addition, *in vitro* study showed that the stem extract from *T. cordifolia* could scavenge superoxide anions as well as hydroxyl radicals (Kumar 2015).

Antioxidants are known as free radical scavengers that reduce cell damage caused by reactive oxygen species (ROS). Reactive oxygen species (ROS) play a role in various health conditions. Antioxidants present in plant-based diets have been associated with their ability to counteract conditions such as cardiovascular disorders and type-2 diabetes mellitus (T2DM). This positive effect is attributed to their capacity to neutralize free radicals within the body (Stanner & Weichselbaum, 2012). Antioxidants interact with free radicals through a range of mechanisms. In the mechanism involving hydrogen atom transfer (HAT), a free radical takes a hydrogen atom from an antioxidant, leading the antioxidant itself to become a radical. Conversely, in the single electron transfer (SET) mechanism, antioxidants contribute electrons to free radicals, resulting in the antioxidant adopting a radical cation (Liang & Kitts, 2014). An example illustrating the assessment of antioxidant capability is the ferric ion-reducing power assay, commonly used to measure the electron-donating ability of antioxidants. This characteristic is particularly important for phenolic antioxidants, through the ability to reduce of Fe^{3+} to Fe^{2+} (Irshad *et al.,* 2012). Another instance of SET is seen in the application of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) experiment. In this case, the unpaired electron within DPPH generates a distinctive deep purple colour with an absorption band at 517 nm. However, when this unpaired electron pairs with another electron due to the intervention of an antioxidant, an alteration in colour occurs, resulting in a pale yellow hue (Sadeer *et al.,* 2020).

Prolonged elevation of blood glucose levels significantly contributes to the production of advanced glycation end-products (AGEs). These AGEs are the result of intricate reactions between carbonyl moieties found in reducing sugars and unbound amino groups in proteins, lipids, or nucleic acids. The formation and accumulation of methylglyoxal (MGO), an active dicarbonyl compound, have been intricately associated with the commencement and advancement of type 2 diabetes mellitus (T2DM). The glycolysis pathway generates the most reactive dicarbonyl molecules, including methylglyoxal (MGO) which is a significant precursor of AGE. (Schalkwijk & Stehouwer, 2020). When investigated *in vitro*, it also induces glycative stress, a condition defined through increasing AGEs resulting in considerable AGEs accumulation in human MSC-derived osteoblasts (Waqas *et al.*, 2022). As a result, MGO and AGEs produced from MGO can have an effect on organs and tissues, possibly altering their structure and functions.

Multiple assays are necessary to show the bioactive compounds potential as a single antioxidant assay cannot accurately predict total antioxidant capacities of a particular compound (Kumar *et al.,* 2018). Hence, this study aimed to show the antioxidant capabilities of methanol and hot water extracts of *T. crispa* and *T. cordifolia* through free radical scavenging capabilities and reducing power. The antiglycation capacity of these extracts also was assessed towards the BSA-MGO complex. This study also intended to identify and quantify the phytochemical compounds that are present in these plant extracts using gas chromatography coupled with mass spectrophotometry (GCMS).

MATERIALS AND METHODS

Chemicals

Aminoguanidine (AG), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, D-glucose, bovine serum albumin (BSA), methylglyoxal (MGO), sodium hydroxide (NaOH), and iron (III) chloride ($FeCl_3$), were acquired from Sigma-Aldrich, USA. Chloroform and methanol were acquired from Fisher Scientific, UK. Sulphuric acid (H_2SO_4) and Dragendorff were purchased from Merck Millipore, USA. Acetic acid glacial was purchased from Avantor Performance, Taiwan. Phosphate buffer solution (PBS) was purchased from Corning, USA.*T. crispa* plant powder was purchased from Shine Tech Solutions, Malaysia and *T. cordifolia* was purchased from Raihan Maju Empire, India.

Plant material extraction procedure

20 g of *T. crispa* plant powder and *T. cordifolia* powder were added separately to 400 mL of 90% methanol. Each mixture was left sitting in the dark at 24 ºC - 25 ºC (room temperature) for 24 hrs. Then, the mixture was filtered through filter papers with pore size of 8 – 12 µm pore size (filtraTECH). The solvent was evaporated using a rotary evaporator. The same procedure was repeated for producing boiled water extracts (100 ℃). The dried extracts were collected and freeze-dried before storing them in a -20 ℃ freezer for further experimental procedures. The extracts were then resuspended with 0.1% PBS before executing further assays.

Detection of saponins

This assay was performed according to Das *et al.,* (2014). Five mL of distilled water was added into a test tube containing 2 mL of the 50 mg/mL PBS-suspended extracts. The mixture was then shaken vigorously for about 10 minutes. Froth formation indicated the presence of saponins. In this test to detect saponins, the froth formation provides insights into their concentration levels. A significant and persistent froth indicates a high concentration of saponins. Moderate froth, which forms but dissipates relatively quickly, reflects a moderate saponin content. In contrast, minimal or no froth suggests a low concentration of saponins in the sample.

Detection of flavonoids

This assay was carried out in line with the protocol by Gul *et al.,* (2017). Two mL of 2 % sodium hydroxide (NaOH) was added to the 50 mg/mL suspended plant extracts. Then 10% of H_2SO_4 was added into the mixture. A yellow colour formation indicated the presence of flavonoids. At low concentrations of flavonoids, the colour change is typically faint and less intense, appearing as a pale yellow or light orange. This subtle colour indicates a lower flavonoid concentration. Conversely, at high concentrations, the colour change is vivid and intense, often resulting in bright yellow or deep orange hues. This strong colour reflects a higher concentration of flavonoids, making it more noticeable and pronounced.

Detection of aglycones (Liebermann's test)

This assay was carried out in line with the protocol by Gul *et al.,* (2017). Two mL of acetic acid and 2 mL of chloroform were added to 1 mL of 50 mg/mL of extracts before adding 2 mL of H_2SO_4. The green colour formation indicated the presence of steroidal aglycones part of glycosides. In the Liebermann's test, the presence of steroidal aglycones is indicated by a colour change to blue-green. A strong and vivid blue-green colour signifies a high concentration of steroidal aglycones, with the intensity and depth of the colour directly correlating to the concentration of steroids in the sample. Conversely, a pale or less intense blue-green colour

suggests a lower concentration of steroidal aglycones, with the colour being less pronounced compared to samples with higher concentrations.

Detection of alkaloids (Dragendorff test)

This assay was performed according to Das *et al.,* (2014). One mL of Dragendorff reagent was added into 2 mL of PBS-suspended 50 mg/mL of extracts in a test tube. Orange colour to brown precipitation indicated the presence of alkaloids. In the Dragendorff test, a strong concentration of alkaloids is indicated by the formation of a deep, intense orange to reddish-brown precipitate. This vivid colour reflects a high concentration of alkaloids in the sample. Conversely, a weaker concentration of alkaloids is shown by a pale or less intense orange to reddish-brown precipitate, indicating a lower concentration of alkaloids.

Detection of terpenoids (Salkowski's test)

This assay was performed according to Shah & Yadav (2015). Two mL of $FeCl_3$ was added to 1 mL of the PBS-suspended 50 mg/mL of extracts in a test tube. Colour changes that ranging from red-brown-violet indicated the presence of phenols. In Salkowski's test, a high concentration of terpenoids is indicated by a deep, vivid red or pink colour in the chloroform layer. This intense coloration reflects a strong presence of terpenoids in the sample. Conversely, a lower concentration of terpenoids results in a pale or faint red or pink color in the chloroform layer, signifying a weaker presence of these compounds.

Detection of phenols (Ferric chloride test)

This assay was performed according to Shah & Yadav (2015). Two mL of $FeCl_3$ was added to 1 mL of the PBS-suspended 50 mg/mL of extracts in a test tube. Colour changes that ranging from red-brown-violet-green indicated the presence of phenols. In ferric chloride test, a high concentration of phenols is indicated by a deep red-brown-violet-green colour. This intense coloration reflects a strong presence of phenols in the sample. Conversely, a lower concentration of phenols results in a pale or faint red-brown-violet-green signifying a weaker presence of phenolic compounds.

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) antioxidant assay

The DPPH antioxidant analysis was performed with minor modifications based on the procedure described by Zulkefli *et al.,* (2013). Control samples containing ascorbic acid were prepared with concentrations ranging from 0.0375 to 0.5 mg/mL. In brief, a mixture comprising 100 µL of 0.1 mM DPPH in methanol was combined with 800 µL of the ascorbic acid standards. Similarly, 100 µL of 0.1 mM DPPH in methanol was mixed with 800 µL of extracts prepared at the same concentrations as the standards. After a 15-mins incubation period, the absorbance was measured at 517 nm. The quantification of the percentage of DPPH inhibition was calculated using following formula:

$$\text{DPPH inhibition}(\%) = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{extracts}}}{\text{OD}_{\text{control}}} \text{ x } 100\%$$

Ferric reducing antioxidant power (FRAP) assay

The assessment of Ferric reduction potential in both extracts was adapted from Jaiswal *et al.,* (2014) with slight modifications. The reagent (FRAP) synthesis involved the addition of acetate buffer with the volume of 20 mL (pH 3.6, 300 mM) to a solution comprising two mL of 2,4,6-Tripyridyl-S-triazine TPTZ with a concentration of 10 mM in Hydrochloric acid (40mM) and two mL of $FeCl_3$ solution (20 mM). Calibration employed ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) across multiple concentrations, spanning from 0.025 to 0.4 mg/mL. The extracts and controls were also prepared in concentrations from 0.05 - 0.4 mg/mL. To initiate the reaction, 300 µL of extracts or controls and 600 µL of distilled water were combined with 1200 µL of the FRAP reagent. Incubation ensued at 37°C for 10 mins, after which the absorbance of samples was measured at 593 nm. The same procedural regimen was replicated for $FeSO_4 \cdot 7H_2O$ to acquire data for constructing the calibration curve. The resultant FRAP values were denoted in terms of mmol Fe^{2+} per mg of the extracts.

BSA-MGO antiglycation assay

BSA-MGO glycation assay was performed according to method adapted from Starowicz & Zieliński (2019). BSA with the concentration of 2 mg/mL and 400 mg/mL MGO were dissociated in PBS (pH 7.4). Samples tubes consisted of 300 µL of BSA, 300 µL of MGO, and 300 µL of extracts ranging from 0.05 to 0.4 mg/mL. A mixture of 300 µL of BSA, 300 µL of MGO and 300 µL of PBS was considered as a negative control in this assay. Positive control was prepared by mixing 300 µL of 1 mol/L aminoguanidine (AG) with 300 µL of MGO and 300 µL of BSA. AG also served as the positive control. These tubes were incubated at 37 ºC for 7 days. PBS was considered as blank, and all absorbance values were deducted with the blank absorbance value before proceeding with calculations. The relative fluorescence unit (RFU) was read using fluorescence with the wavelength of (excitation, 340 nm; emission, 420 nm). The percentage of glycation inhibition was calculated

using the following formula:

$$\text{Glycation inhibition (\%)} = \frac{\text{RFU}_{\text{control}} - \text{RFU}_{\text{extracts}}}{\text{RFU}_{\text{control}}} \text{ x } 100\%$$

Gas Chromatography coupled with Mass Spectrophotometry analysis (GC/MS)

GC-MS analysis was performed according to Samling *et al.,* (2021) using a Shimadzu GCMS-QP2010 Plus (Shimadzu, Japan). The GC separation was carried out using BPX-5 capillary column (Trajan Scientific and Medical, Australia) (30 m x 0.25 mm). The column was coated with fused silica material, phenyl polysilphenylene-siloxan (5%) with film thickness of 0.25 µm. Electron impact with ionization energy of 70 eV with interface temperature of 250℃ were applied together with scan mass ranging from 28-400 *m/z*. Helium gas was used as the carrier gas at a flow rate of 1.0 mL/min. The volume injection was 1.0 µl with a split ratio of 20:1. The identification of phytochemical compounds responsible for antioxidant activities of both methanolic and water extracts were made using the NIST-17 mass spectral library incorporated in the system.

Statistical analysis

The statistical analyses were performed using IBM SPSS Statistics Data Editor (Ver. 25). All the experiments were done in triplicates (n=3) and descriptive measures were calculated to retrieve mean as well as standard deviation (s.d) values. One-way ANOVAs were run and Dunnett's test was executed to compare the difference between the control mean with the samples while Tukey's test was implemented to compare differences between the sample means. The results were tabulated and graphed accordingly with mean ± standard deviation values.

RESULTS AND DISCUSSION

Qualitative phytochemical screening

The qualitative phytochemical screening of *T. crispa* and *T. cordifolia* revealed that saponin, flavonoids, glycosides, alkaloids, terpenoids, and phenols were present in both extracts as listed in **Error! Reference source not found.**. Saponins are glycosylated triterpenes and steroids, and they are the secondary metabolites in plants that can form soap-like foams in aqueous solution. In this study, saponin was detected in all of the extracts with the highest concentration in methanolic *T. crispa* followed by methanolic and aqueous *T. cordifolia,* and the least amount in aqueous of *T. crispa*. Flavonoids, a plant secondary metabolite with a polyphenolic structure were also detected with the highest concentration in aqueous *T. crispa,* followed by aqueous *T. cordifolia,* methanolic *T. crispa* and methanolic *T. cordifolia*. Liebermann's test that determines the presence of aglycones revealed that aqueous *T. cordifolia* had the highest concentration of aglycones when compared to aqueous *T. crispa,* methanolic *T. crispa* and methanolic *T. cordifolia.* Alkaloid is a secondary plant metabolite composed mainly of nitrogen and various pharmaceutical importance. Its highest concentration was found in methanolic *T. crispa,* and aqueous extracts of both *T. crispa* and *T. cordifolia* while the least alkaloid was shown by methanolic *T. cordifolia.* Salkowski's test revealed that methanolic and aqueous *T. crispa* possessed higher concentration of terpenoids when compared to methanolic and aqueous *T. cordifolia.* Methanolic extracts for both species revealed the highest concentration of phenolic content, followed by their aqueous extracts.

Table 1. Phytochemical screening analysis for *T. crispa* and *T. cordifolia*

Phytochemical tests	*T. crispa* (water)	*T. cordifolia* (water)	*T. crispa* (methanol)	*T. cordifolia* (methanol)
Saponin (Frothing test)	+	++	+++	++
Flavonoids (Alkaline reagent test)	+++	++	+	+
Aglycones (Liebermann's test)	+	+++	+	+
Alkaloids (Dragendorff' s test)	++	++	++	+
Terpenoids (Salkowski's test)	+++	+	+++	++
Phenols (Ferric chloride test)	++	+	+++	+++

A plus sign (+) denotes the concentrations of polyphenols detected in the extracts. (+++) is high, (++) medium while (+) is weak.

Phytochemical compounds otherwise known as secondary metabolites are compounds with numerous active compounds (Mendoza & Escamilla 2018). Phytochemical screening aids in the search for bioactive compounds that may be employed in the production of effective medications. For instance, phenolic compounds serve as antioxidants by interacting with free radicals. The mechanism of antioxidant activity involves either hydrogen atom transfer, single electron transfer, sequential proton loss electron transfer, and transition metal chelation (Zeb, 2020). Flavonoids specifically can serve as antioxidants (Kalita *et al.,* 2013). All extracts also reveal the presence of steroidal aglycones of glycosides which are distributed enormously in the plant kingdom. Aglycones had been reported to possess anti-inflammatory, antipyretic, anti-microbial, and anti-rheumatic properties (Bartnik & Facey 2017). Terpenoids are a class of compounds based on isoprene units, which are naturally derived from mevalonic acid (Jones *et al.,* 2010). As a result of their capacity to regulate glucose metabolism and hence reduce blood glucose levels, pentacyclic triterpenoids and dammarane terpenoids have

been reported to have antidiabetic properties (Ghani 2020). Alkaloids are secondary metabolites that were originally defined as pharmacologically active nitrogen-based compounds. Alkaloids had been reported to possess an analgesic effect through morphine while cocaine served as a stimulant of the central nervous system (CNS) and local anaesthetic (Richard *et al.,* 2013). Saponin is an enormous group that consists of amphiphilic glycosides of steroids and triterpenes found in plants and certain marine creatures. Earlier investigations propose that saponins might reduce cholesterol levels by creating an insoluble compound with cholesterol, obstructing its absorption within the intestines. Additionally, certain saponins enhance the expulsion of bile acids, an indirect approach to cholesterol reduction, or undergo hydrolysis by intestinal bacteria into diosgenin, potentially yielding advantageous outcomes. (Murphy *et al.,* 2017).

Antioxidant activities of *T. crispa* and *T. cordifolia*

The percentage of DPPH inhibition for methanolic *T. crispa* was between 51.83 ± 1.57% to 79.03 ± 0.66%. Meanwhile, methanolic *T. cordifolia* showed DPPH inhibition between 53.33 ± 0.53% to 81.3 ± 0.28%. Aqueous *T. crispa* showed DPPH inhibition range between 12.82 ± 5.56% to 42.17 ± 3.50% while aqueous *T. cordifolia* showed inhibition with a range between 19.65 ± 4.74% to 72.80 ± 0.66%. All the extracts were significant to their respective ascorbic acid standard at p<0.01 except for 0.5 mg/mL methanolic *T. cordifolia*, which was significant at p<0.05 as shown in **Error! Reference source not found.**

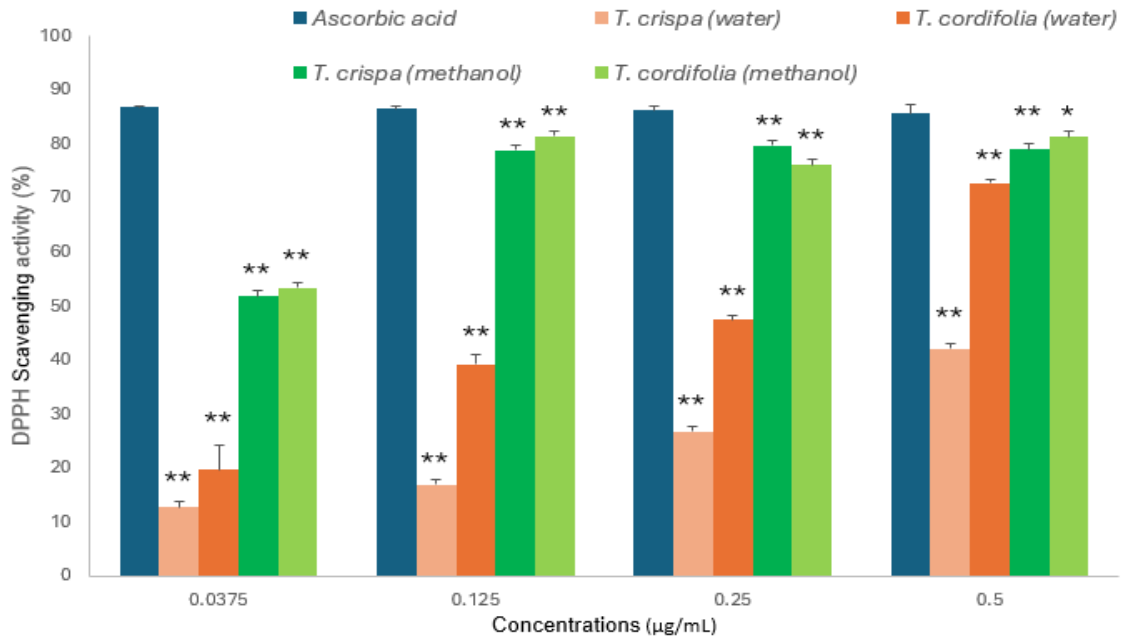


Fig. 1. The mean percentage ± standard deviation values (n=3) of DPPH scavenging activity (methanol and water extracts). Significant values are implied by *p<0.05 as well as **p<0.01, denoting comparison with the corresponding ascorbic acid control through Dunnet's post-hoc test.

The methanolic extract was shown to have stronger free radical scavenging activity than water extracts, consistent with prior research conducted by Ibahim *et al.,* (2011) as shown in Figure 1.The water extract of 0.5 mg/mL *T. crispa* in this study showed a comparable DPPH scavenging activity percentage (42.17%) to 0.5 mg/mL with *Amaranthus spinosus* (44.75%) reported by Baral (2010). *Amaranthus spinosus,* which is widely used in Ayurveda practices also had been reported to exhibit antioxidant properties as well as containing minerals and vitamins (Sarker & Oba 2019). A previous study showed that *T. crispa* stem extract comprised magnoflorine and apigenin, while its root extract contained magnoflorine. Magnoflorine (alkaloid) and apigenin (flavonoid) have hydroxyl groups that contribute electrons to reduce DPPH radicals (Zulkefli *et al.,* 2013). Furthermore, the study also revealed that *T. crispa* leaves contain a high concentration of phenolic chemicals, which serve as hydrogen donors and have been widely reported to have antioxidative properties.

The notable DPPH scavenging efficacy observed in methanolic extracts can potentially be attributed to multiple factors. Polyphenols, due to their inherent chemical properties, exhibit enhanced solubility in organic solvents of lower polarity than water. This, coupled with the polar attributes of polyphenols themselves, might contribute to the observed phenomenon (Haminiuk *et al.,* 2014; Tobgay *et al.,* 2020). Additionally, the interplay between DPPH and antioxidants is intricately influenced by the specific chemical makeup of the antioxidants (Ulewicz & Wesolowski, 2019). For example, the antioxidant functionality of flavonoids is predicated upon the arrangement and substitution pattern of their hydroxyl groups. An optimal radical scavenging activity

necessitates the presence of a 3,4-orthodihydroxy structure within ring B and a 4-carbonyl group within ring C. The introduction of a 3-OH group and 5-OH groups within ring C, culminating in a catechol-like configuration, further augments the antioxidant performance of flavonoids (Wojdyło *et al.,* 2007).

The FRAP activity of both methanolic and water extracts in this study showed an increased trend in a dose-dependent manner as shown in **Error! Reference source not found.**. Methanolic *T. crispa* showed a reducing capacity with the range of 0.0239 ± 0.00 to 0.1322 ± 0.00 mmol Fe^{2+}. Meanwhile, methanolic *T. cordifolia* showed a reducing power with a range between 0.0201 ± 0.002 to 0.1300 ± 0.006mmol Fe^{2+}. Aqueous *T. crispa* showed a reducing power ranging from 0.0072 ± 0.00 to 0.0404 ± 0.00 mmol Fe^{2+} while aqueous *T. cordifolia showed a reducing power* from 0.0170 ± 0.00 to 0.0977 ± 0.00 mmol Fe^{2+}.

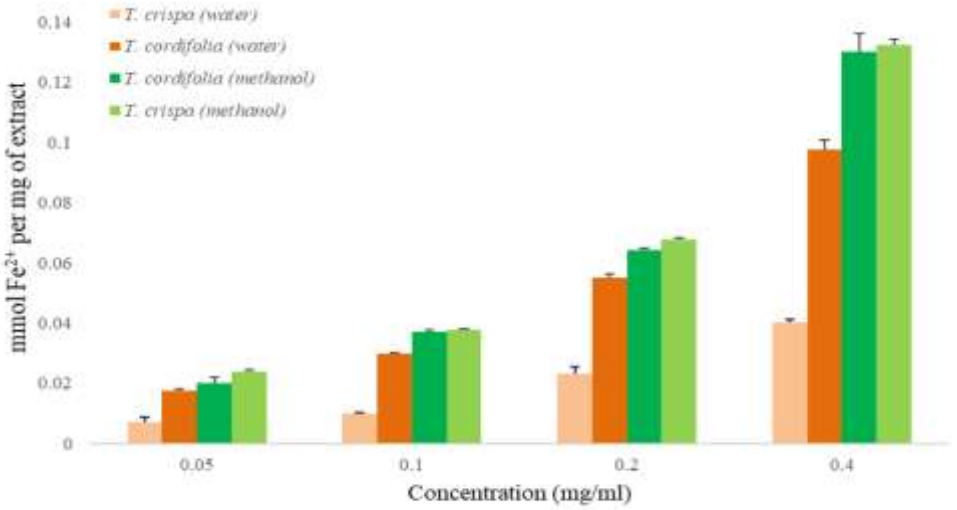


Fig. 2. Mean values of FRAP values ± standard deviation (n=3) expressed as mmol Fe^{2+} per mg extract (both methanolic and water extracts).

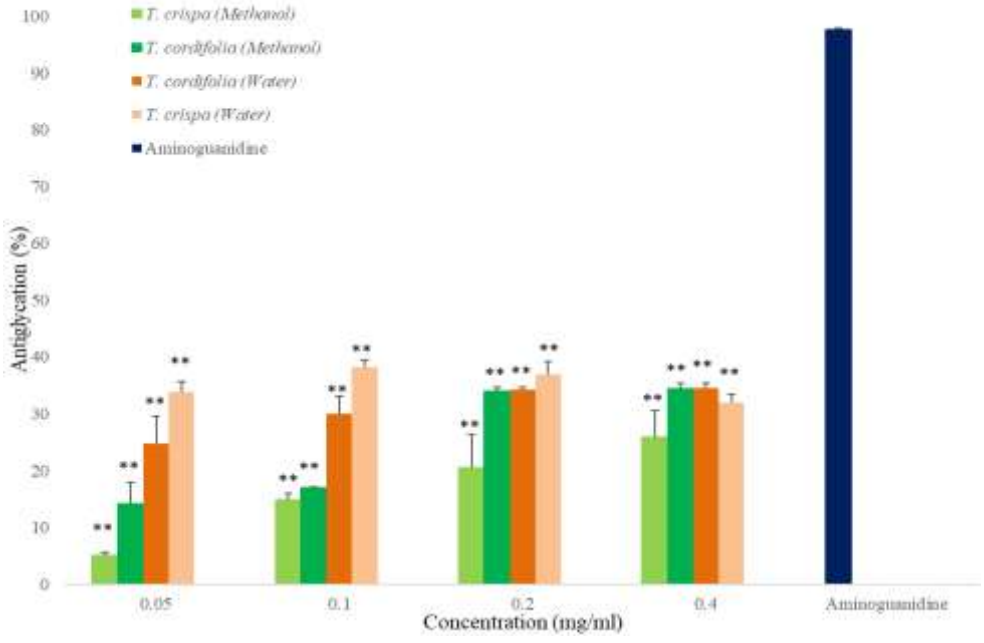


Fig. 3. Antiglycation activity (%) of *T.crispa* and *T.cordifolia (*methanolic and water extracts) towards BSA-MGO ± standard deviation (n=3). Significant values are implied by *p<0.05 as well as **p<0.01, denoting comparison with the Aminoguanidine control, measured through Dunnet's post-hoc test.

The FRAP assay measures antioxidant power by reducing ferric-tripyridyl triazine (Fe^{3+}-TPTZ) to a deep blue colour ferrous-tripyridyl triazine (Fe^{2+}-TPTZ) complex with absorption being measured at 593 nm (Mfotie & Emmanuel, 2021). The FRAP reaction was carried out in an acidic condition with pH of 3.6 to maintain iron solublity as the reaction at low pH reduced the ionisation potential that promoted hydrogen atom transfer and enhanced the redox potential, which was the major reaction mechanism (Cerretani & Bendini, 2010). In the current study, ferum sulphate heptahydrate ($FeSO_4 \cdot H_2O$) was used as standard and the result was expressed as mmol Fe^{2+} per mg of extract.

For this assay, methanolic *T. crispa* was identified to possess higher reducing power properties compared to other extracts. A lower IC_{50} value from reducing power assay suggests a more efficient reducing power capability. In this assay, the oxidation chain reaction is terminated through an electron donor which reduces oxidised intermediates into their stable forms (Lee *et al.,* 2013).

The results of the BSA-MGO assay are shown in **Error! Reference source not found.**. The highest antiglycation inhibition observed at 0.4 mg/mL was 38.52 ± 2.31%, through methanolic *T. cordifolia,* followed by aqueous *T. cordifolia* (36.9 ± 1.516%), methanolic *T. crispa* (33.83 ± 1.327%), and aqueous *T. crispa* (31.96 ± 1.803%) as shown in Fig. 3. In this system, AG prevented the synthesis of fluorescent AGEs via 97.6%. BSA-MGO antiglycation assay measured the capacity of the extracts to reduce antiglycation between protein (BSA) and MGO. Despite showing significant scavenging and reducing power properties, both methanolic and aqueous extracts did not show great antiglycation properties. The antiglycation results from this current study suggest that methanolic and aqueous extracts of *T. crispa* and *T. cordifolia* are capable of scavenging oxidants rather than inhibiting glycation site proteins.

IC_{50} values for each antioxidant assays

Half maximal inhibitory concentration (IC_{50}) values for all the extracts have been determined for each antioxidant assay. For the DPPH assay, IC_{50} represents the concentration needed to scavenge at least 50% of the initial DPPH radicals. Meanwhile, IC_{50} in FRAP assay indicates the concentration required to reduce 50% of the $Fe2^{+}$. For the BSA-MGO assay, IC_{50} represents the concentration needed to inhibit 50% of the glycation between BSA and MGO. The IC_{50} values of the extracts are tabulated in **Error! Reference source not found.**.

Table 2. IC_{50} values for DPPH and FRAP values

Antioxidant assays	IC_{50} values			
	T. crispa (methanol) mg/mL	*T. cordifolia* (methanol) mg/mL	*T. crispa* (water) mg/mL	*T. cordifolia* (water) mg/mL
DPPH scavenging activity	0.04±0.00	0.04±0.00	0.60±0.04	0.34±0.00
FRAP	0.19±0.00	0.20±0.00	0.67±0.00	0.26±0.00

The values represented is mean ± standard deviation (n=3). The lowest IC_{50} for DPPH and FRAP exhibited by methanolic *T. crispa* and *T. cordifolia*.

The IC_{50} values for methanolic extracts were much lower (*T. cordifolia,* 0.04 mg/mL; *T. crispa,* 0.04 mg/mL) when compared to the DPPH scavenging activity of methanolic extract of orange peel (1.401 mg/mL) and flesh (1.710 mg/mL) as reported by Park *et al.,* (2014). This suggests that both methanolic *T. crispa* and *T. cordifolia* have higher antioxidant capabilities in terms of free radical scavenging when compared to orange. Furthermore, the current study revealed the methanolic extract of *T. crispa* (0.04 mg /mL) had lower IC_{50} than methanolic extract (stem) *T. crispa* (0.118 mg/mL) as reported by Zulkefli et al. (2013). This research further elucidated that the methanolic and water extracts of *T. cordifolia* had even lower IC_{50}, 0.04 mg/mL and 0.3 mg/mL respectively than previously reported by Ilaiyaraja & Khanum (2011). In their study (laiyaraja & Khanum, 2011), both methanolic extracts (leaf, 0.54 mg/mL ; stem 0.74 mg/mL) and water extracts (leaf, 1.22 mg/mL ; stem, 1.79 mg/mL) possess higher IC_{50} than *T. cordifolia* reported in this study. All these findings suggest that utilising plants as a whole may exhibit higher antioxidant potential than using certain parts of the plants.

The FRAP activity of both methanolic and water extracts in this study showed an increased trend in a dose-dependent manner (Figure 2). The IC_{50} values of reducing power for these plant extracts decreased in the following sequence, as shown in Table 3: aqueous *T. crispa* > aqueous *T. cordifolia* > methanolic *T. cordifolia* > methanolic *T. crispa.* Methanolic extracts (*T. crispa,* 0.19 mg/mL ; *T. cordifolia*, 0.19 mg/mL) had the lowest IC_{50} values (i.e., the concentrations of samples required to reduce power activity by at least 50%), followed by water extracts (*T. cordifolia,* 0.26 mg/mL ; *T. cordifolia,* 0.67 mg/mL).

Gas chromatography Mass Spectrophotometry (GC-MS)

Polyphenols, also referred to as phenolic compounds, constitute a versatile spectrum of metabolites arising from plant secondary metabolism. Distinguished by at least hydroxyl groups linked to a benzene ring, they display significant antioxidant activities (Daglia, 2012). By engaging with free radicals, phenolic compounds (PCs) function as antioxidants. The underlying mechanism of their antioxidant action encompasses HAT, SET, sequential proton loss electron transfer, and transition metal chelation (Zeb, 2020). The GC-MS analyses conducted this study have revealed a multitude of phytochemical compounds detected across both methanolic and aqueous extracts of *T. crispa* and *T. cordifolia* that are responsible for antioxidant activities as shown in **Error! Reference source not found.** and **Error! Reference source not found.**.

Fig. 4. The GC-MS chromatogram. (a) GC-MS chromatogram for methanolic *T. cordifolia*.(b) GC-MS chromatogram for methanolic *T. crispa*.

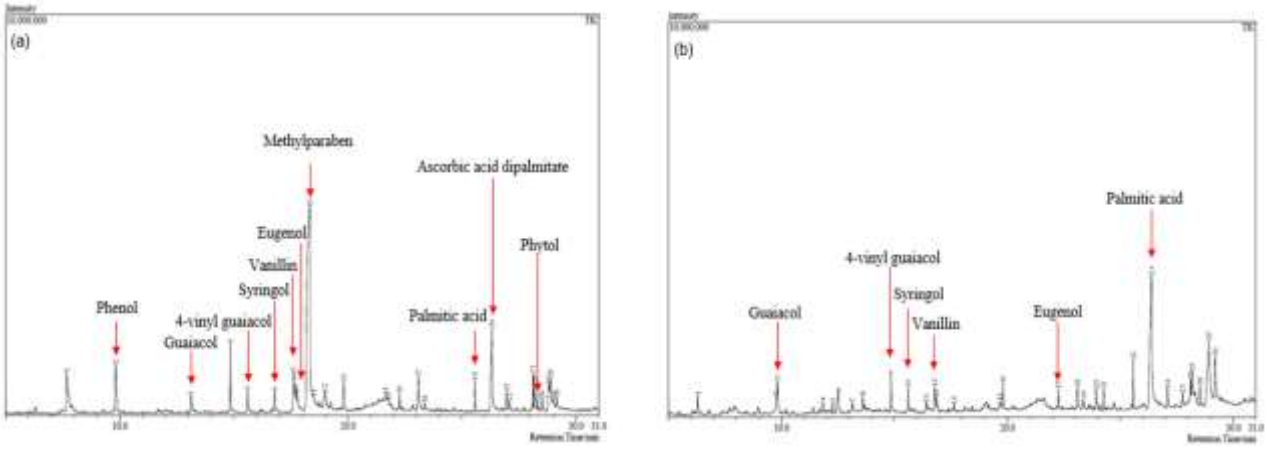
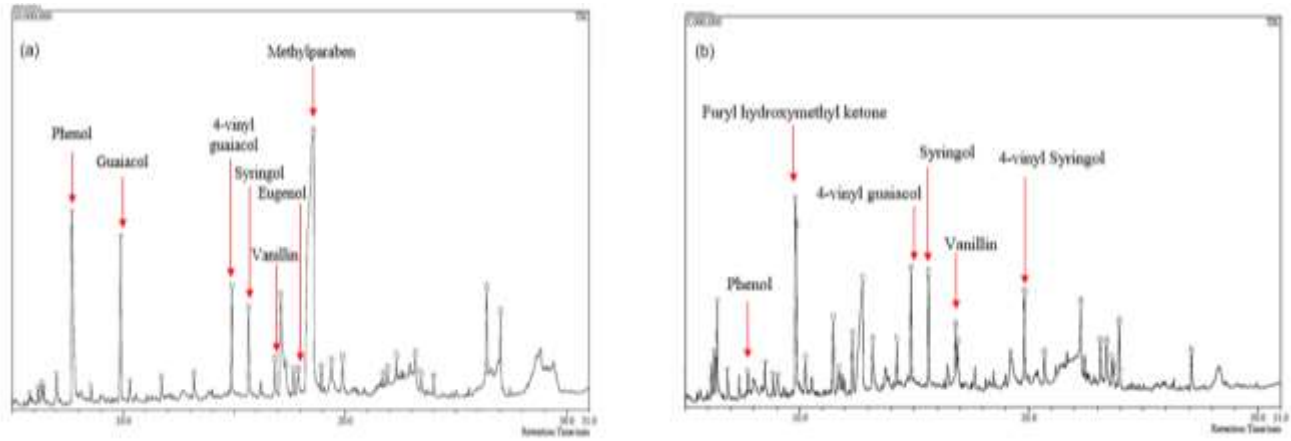


Fig. 5. The GC-MS chromatograms. (a) GC-MS Chromatoram for aqueous *T. cordifolia*. (b) GC-MS Chromatogram for aqueous *T. crispa.*



Phenols, renowned for their adeptness at chelating metals, interact with free radicals via HAT or SET mechanisms (Zeb, 2020). FTIR investigation has revealed the presence of 4-vinyl guaiacol, which features hydroxyl and methoxy functional groups. This feature highlights its comparable antioxidant efficacy regarding commercially established antioxidants (Azadfar *et al.,* 2015). Eugenol, a noteworthy phenylpropanoid detected in both methanolic *T. crispa* and *T. cordifolia* extracts, notably thrives as a principal component within clove essential oil and exhibits apparent antioxidant potential (Silva *et al.,* 2018). Its potency extends to the complete cessation of both iron and Fenton reagent-mediated lipid peroxidation (Nagababu *et al.,* 2010). Moreover, eugenol inhibits 96.7% (R^2=0.9319) lipid peroxidation in a linoleic acid emulsion, possessing the highest antioxidant compound with radical-scavenging activity when compared to Trolox, butylated hydroxytoluene and other antioxidant standards (Gülçin, 2011). Syringol, recognized for its antioxidant attributes, wherein its ability to scavenge DPPH radicals, neutralize ABTS radical cations, and manifest ferric reducing antioxidant power (FRAP) is emphasized by the study of (Loo *et al.,* 2008).

Meanwhile, palmitic acid, one of the fatty acid compounds found in methanolic extracts of both *T. crispa* and *T. cordifolia* had been shown to possess biological activities including antioxidants (Siswadi & Saragih, 2021). Furthermore, guaiacol is a commercial antioxidant (Anouar *et al.,* 2009) and has been widely used in food preservation and pharmaceuticals (Azadfar *et al.,* 2015). Furthermore, the antioxidant effects of certain polyphenols are linked to the existence of the guaiacol functional group (Gao *et al.,,* 2021). Moreover, methanolic and aqueous extracts of *T. cordifolia* revealed the presence of methylparaben. By using using voltametric techniques, methylparaben was shown to possess smaller oxidation potentials than unsaturated fatty acids such as oleic, making it one of many preservatives that protect compounds against oxidative damage (Michalkiewicz, 2013). Meanwhile, vanillin, a benzaldehyde was found in all extracts. A study showed that vanillin has significant activity in the ABTS$^{\bullet+}$ scavenging assay through a self-dimerization mechanism and also through oral administration, it improved the antioxidant level in mice plasma (Tai *et al.,* 2011). Phytol, a diterpene that has been reported only in methanolic *T. cordifolia* (Fig.4a) *had* shown antioxidant properties in both non and preclinical test systems and has been suggested as a candidate for treatment for oxidative stress mediated diseases (Costa *et al.,,* 2016). In vitro study showed that phytol had a significant antioxidant property by removing hydroxyl radicals as well as nitric oxide and reducing the synthesis of thiobarbituric acid reactive substances (TBARS). (Santos *et al.,* 2013).

Furyl hydroxyl methyl ketone (Fig.5b), which belongs to furans and furanone compound was detected in aqueous *T. crispa*. This compound was also detected in *Gelam* honey and is known to possess antioxidant properties as reported by Ismail *et al.,* (2021). Furthermore, furans and furanone have been studied for their pharmacological activities which include anti-inflammatory and antioxidants (Husain *et al.,* 2019). Aqueous *T. crispa* also revealed the presence of 4-vinyl syringol, which has been studied for its antioxidative properties. For instance, 4-vinyl syringol is known for its scavenging properties, an antioxidant against the oxidation of lipids and protein Terpinc *et al.,* (2011).

As this study is looking at antioxidants and their potential benefits in combating oxidative stress, it is limited by its focus on methanolic and aqueous extracts, which may not capture the full spectrum of bioactive compounds present in *T. crispa* and *T. cordifolia*. To gain a more comprehensive understanding of the plants' potential benefits, future research should explore extracts using additional solvents. Furthermore, while the study demonstrates promising antioxidant activities, it is crucial for future research to conduct i*n vivo* validation to confirm these effects and assess their practical relevance in a living organism.

CONCLUSION

The antioxidant activity determinations performed for this study showed that methanolic extracts of the plants react successfully to radical sources, with methanolic *T. cordifolia* as the most efficient free radical scavenger and anti-glycating agent while methanolic *T. crispa* effectively reduced the Fe^{3+} to Fe^{2+}. Based on GC-MS results, the investigated methanolic and aqueous extracts revealed compounds as previously reported in earlier studies such as phenol, guaiacol, eugenol and syringol. Keeping in consideration with the research derivation, it can be concluded that *T. crispa* and *T. cordifolia* have the potential to reduce T2DM progression as well as T2DM-related diseases as complementary diet alongside with prescribed drugs. However, further study on the plant's toxicity as well as *in-vitro* study towards some of the important markers related to T2DM-related diseases might be helpful to deepen our understanding towards the potential that these plants might possess, before incorporating them into the diet.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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