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Pro-oxidant *Tetrapleura tetraptera* polyphenol extract parallels quercetin as an *in vitro* cytotoxic agent in human liver cancer cells

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ABSTRACT

Tetrapleura tetraptera (TT) fruits, also called aidan fruits, are traditionally used in Africa and other parts of the world as a spice in foods and in treating diseases such as arthritis, diabetes, infections and cardiovascular diseases but have remained largely underutilized. To find new ways of utilizing TT dry fruits, this study produced pro-oxidant polyphenols from it by applying the combined effects of high-power, low frequency ultrasound and the Fenton-like mechanism. The efficacy of the pro-oxidant polyphenols as cytotoxic agent was tested in human liver cancer cells (HepG2), evaluating its potential use as an anti-cancer remedy. The Folin Ciocalteau, DPPH, ABTS and cellular antioxidant activity (CAA) assays were used to determine total phenolic content (TPC) and antioxidant property of extracts. Pro-oxidant property of extracts was confirmed using lipid oxidation assays that measured malondialdehyde (MDA) levels and peroxide value (PV) in samples. It was found that TT polyphenol (TTP) extract treated with $FeCl_3$ (TTP-$FeCl_3$) showed poor antioxidant properties evident in its ability to promote lipid oxidation (i.e. increasing MDA levels from 0.17 to 2.53 μmol g^{-1} and PV from 0.40 to 1.16 meq O_2 kg^{-1} fat). It also induced cytotoxic effects in HepG2 cells. From LC-ESI-MS analysis, the major phenolic compounds and their concentrations in the TTP-$FeCl_3$ extract were kaempferol-3-*O*-rutinoside (23.75 μg g^{-1}), 4-*O*-methylgallic acid (19.05 μg g^{-1}), chlorogenic acid (16.22 μg g^{-1}), rutin (16.15 μg g^{-1}), quercetin-3-*O*-glucuronide (13.19 μg g^{-1}), cyanidin-3-*O*-galactoside (12.58 μg g^{-1}), orientin (10.99 μg g^{-1}), isorhamnetin-3-*O*-glucoside (10.11 μg g^{-1}) and gallic acid (10.47 μg g^{-1}). This study showed that prolonged high-power, low-frequency ultrasonication in combination with $FeCl_3$ treatment can be used to produce pro-oxidant polyphenols capable of eliciting cytotoxic effects in HepG2 cells *in vitro*. Exploring targeted delivery of TTP pro-oxidants with cytotoxic properties has great potential in disease management.

1. Introduction

For many decades, polyphenols have been known to be healthful and this has been attributed to their antioxidant properties (Belščak-Cvitanović et al., 2018; Rice-Evans et al., 1997). However, studies have also shown that polyphenols can also exhibit pro-oxidant properties in living things (Bacchetti et al., 2020; Giordano et al., 2020; Lambert & Elias, 2010; Nowak et al., 2022). In living cell lines, pro-oxidant polyphenols have been shown to inhibit cellular proliferation (Giordano et al., 2020; Lambert & Elias, 2010; Lu et al., 2021). Considering certain mechanisms in antioxidant systems as influenced by redox potential of cellular environments, pro-oxidant activity of polyphenols is needed to maintain homeostasis and has also been an inducer of cellular apoptosis through enzyme inactivation *via* S-glutathionylation (Chedea et al., 2021; Forester & Lambert, 2011; Lu, 2013; Pérez-Torres et al., 2017). Thus, the ability of polyphenols to act as pro-oxidants by virtue of their structure (Kessler et al., 2003; Maurya & Devasagayam, 2010; Yordi et al., 2012), concentration (Eghbaliferiz & Iranshahi, 2016; Zhou & Elias, 2013), pH of their immediate environment (Zhou & Elias, 2013), and their ability to deprotonate and form complexes with metal ions (Lemańska et al., 2001; Spiegel et al., 2023) is an opportunity to be exploited positively considering theragnostic applications in oncology.

In this study, ultrasound assisted extraction of polyphenols in water is considered an appropriate environmentally-friendly approach to produce polyphenols with high pro-oxidant properties. This

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consideration is based on previous studies that have shown that low-frequency, high-power ultrasound produces energy-dense acoustic cavitation that has the ability to induce autolysis of water molecules in a low-density environment to generate free hydroxyl radicals that oxidize polyphenols (Dzah, Duan, Zhang, Boateng, et al., 2020). As low frequency enhances acoustic cavitation, high power ensures that enough energy is produced in the extraction medium and low density medium favours the efficient transfer of ultrasound power within the medium, hence reducing power attenuation significantly (Dzah & Dzigbor, 2023). In the absence of added antioxidants, ultrasound assisted extraction using distilled water as the sole solvent is expected to deprotonate polyphenols, especially in a basic physiological environment (i.e. pH between 6 and 7).

The plant material, *Tetrapleura tetraptera* (TT) was chosen to be used as a reliable source of polyphenols because it has been underutilized and limited to usage in traditional medicine and food production with minimal research focus currently (Kemigisha et al., 2018). *T. tetraptera* is a flowering plant common in Western and Central Africa. The plant is called *prekese* and *uhio* in Ghana and Nigeria, respectively, and has been adored for its numerous uses. The fruit is also commonly known as *aidan fruit* in many parts of the world. It is noted for its sweet fragrance and is used as spice in dishes and beverages and its bark, leaf and fruits have been used for medicinal purposes. *T. tetraptera* fruits have been recognized for their high nutritional value (~64 % carbohydrates, 289 mg g^{-1} potassium, 200 mg g^{-1} calcium, 342 mg g^{-1} manganese, and 5 mg g^{-1} vitamins) and phytochemical composition (phenolic acids, flavonoids, saponins, tannins and alkaloids) (Mensah et al., 2024). It has been reported to have significant medicinal value due to its biological significance as it possesses antioxidant, anti-proliferative, anti-inflammatory, antimicrobial, antidiabetic, and anti-parasitic properties (Dzah, 2022, 2023). It is therefore commonly used to treat diseases such as diabetes, cancer, hypertension, immune disorders, malaria and many other diseases. Earlier studies have also extracted polyphenols from TT, tested its biological activity and its phenolic composition (Dzah, 2022, 2023). Given its rich phytochemical content, it is important to further explore ways to utilize its bioactive compounds through research and innovation.

The main goal of this study was to produce pro-oxidant polyphenols from TT fruits using the combined effect of ultrasound cavitation and the Fenton-like reaction. This is the first study that has employed the deliberate use of acoustic energy to produce pro-oxidant polyphenol extracts, especially from TT fruit. The study explored the role of $FeCl_3$ as a source of Fe^{3+} for the induction of Fenton-like reaction for free radical production as well as that of hydroxyl radical (OH*) generation from water breakdown during sonication. The phenolic content, phenolic profile, pro-oxidant activity and biological properties of extracts were assessed using both chemical and cellular *in vitro* assays. The cytotoxic effect of polyphenol extracts in human liver cancer cells was also investigated.

2. Materials and methods

2.1. Materials

2.1.1. T. tetraptera fruit powder preparation

T. tetraptera dry fruits were purchased from the Ho central market in the Volta region of Ghana. Fruits of dark-brown colour, measuring about 20 cm in length and 4 cm in width were considered mature as those of smaller dimension looked light-weighted and light-brown and contained underdeveloped seeds. The whole fruits were cleaned with moist cotton cloth and oven-dried at 50 °C for 12 h. Dry fruits were cut into smaller chunks and pulverized into fine powder using a laboratory grade steel blender. The powder was passed through an 80-mesh laboratory sieve, put in a zip-lock plastic pouch and stored at −20 °C for a maximum of 20 days for further use.

2.1.2. Reagents and chemicals

Folin Ciocalteu's reagent for total phenolic content determination, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), ascorbic acid, MTT, DMSO, RPMI, HepG2 cells and other reagents and chemicals such as $FeCl_3$ and ascorbic acid were purchased from Sigma Aldrich (Shanghai, China).

2.2. Methods

2.2.1. Ultrasound-assisted polyphenol extraction

According to single-factor experiments and outcomes of optimizations from previous studies on ultrasound-assisted extraction (UAE), *T. tetraptera* polyphenols (TTP) were extracted in distilled water using ultrasound-assisted method using the following conditions- frequency (20 kHz), power (300 W), time (45 min), temperature (50 °C) and solvent-to-material ratio (SMR) (80 mL g^{-1}) (Dzah, 2022; Dzah, Duan, Zhang, Golly, et al., 2020). From earlier experiments, low frequency-high power UAE at high SMR gave the best polyphenol yield. Also, UAE at temperatures above 50 °C and 45 min resulted in significant loss of antioxidant activity of extracts. Sonication was done using an ultrasonic bath fitted with a bidirectional benchtop water pump to control water level and temperature (Fig. 1). Hot water extraction (HWE) in boiling water (100 °C) for 15 min at SMR of 80 mL g^{-1} was done to serve as control extraction method.

2.2.2. Polyphenol purification

Polyphenol extracts were purified using the AB-8 macroporous resin according to an earlier method (Dzah et al., 2020). Fig. 2 gives a summary of the methodology. Extracts pretreated with $FeCl_3$ and ascorbic acid were purified the second time to wash out excess chlorine as HCl (Fig. 3) as well as ascorbic acid with water. All purified extracts were stored at −20 °C for a maximum of 5 days for further analysis and at −80 °C for longer storage.

2.2.3. Pretreatment of polyphenol extracts to induce anti-/ pro-oxidant properties

After purification of extracts, they were pretreated to modify their chemical properties using ascorbic acid and $FeCl_3$. Polyphenol extract (50 mL) was mixed with $FeCl_3$ (2 mL 0.1 M) and sonicated at 20 kHz for 10 min to produce TTP-$FeCl_3$ extract. The process was repeated using ascorbic acid (2 mL 0.75 M) to produce TTP-Ascorbic acid. Through the reaction of Fe^{3+}, it was expected that in the presence of free hydroxyl radicals and other reactive oxygen species (ROS), phenol radicals with pro-oxidant properties would be produced (Fig. 3). Using ascorbic acid was to curtail the degradative effect of ultrasound-induced ROS on

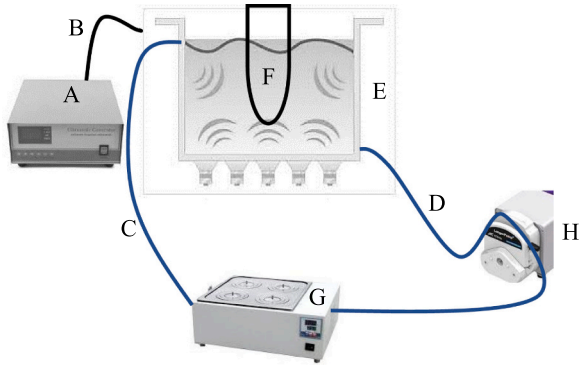

Fig. 1. Setup for ultrasound-assisted extraction of TTP. A = ultrasound generator; B = power cable; C = water out of sonicator; D = water into sonicator; E = sonicator; F = sample; G = water bath with temperature control; H = bidirectional bench pump.

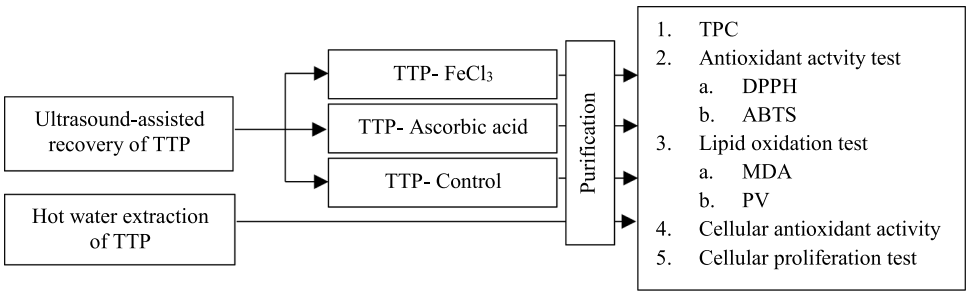

Fig. 2. Summary of methodology.

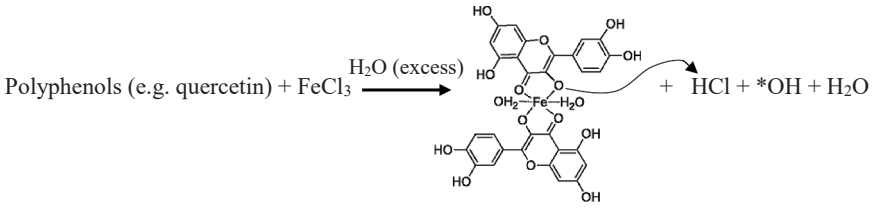

Fig. 3. Phenol-iron complexation mechanism resulting in the oxidation of phenols.

polyphenols, thereby maintaining their antioxidant properties (Dzah et al., 2020). Extract that was not pretreated with $FeCl_3$ and ascorbic acid served as control (TTP-Control).

2.2.4. Total phenolic content determination

Total phenolic content (TPC) was determined according to the Folin Ciocalteu's method (Dzah, 2023). A 10-fold dilution of Folin reagent was made and 2 mL was added to 1 mL of extracts and mixed. Na_2CO_3 solution (3 mL, 25 %) was added to the mixture after which they were incubated at 40 °C for 45 min in the dark. The absorbances of samples were measured spectrophotometrically at 765 nm, using gallic acid and distilled, deionized water as standard and blank, respectively. TPC was estimated using a gallic acid calibration curve (R = 0.998). Results were expressed as milligram of gallic acid equivalents per gram dry weight (mg GAE g^{-1} DW).

2.2.5. Antioxidant activity determination

2.2.5.1. DPPH and ABTS assays. Although the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays are used to measure antioxidant activity of substances through ROS scavenging ability, it is expected that pro-oxidants would lack the ability to scavenge ROS. Thus, while antioxidants reduce DPPH and ABTS radicals and decrease absorbance of reaction mixture, pro-oxidants have no reducing effect and the absorbance of reaction mixture increase instead. As phenolic compounds reduce hydroxyl radicals, they were expected to produce phenol-radicals and act as ROS. The failure of extracts to exhibit ROS-scavenging ability is considered pro-oxidant activity. The DPPH and ABTS assays followed methods described in earlier studies (Khan et al., 2023).

2.2.5.2. Lipid oxidation determination using the malondialdehyde (MDA) and peroxide value (PV) assays. To determine the pro-oxidant activity of extracts, they were mixed with vegetable oil (5 mL: 50 mL; extract: oil) and allowed to stand in a 150 mL conical flask at 70 °C for 2 h. The level of the lipid oxidation product, MDA was used to determine the pro-oxidant activity of extracts. To extract MDA, 1 g of treated vegetable oil was added to 5 mL 50 % ethanol and vortexed for 2 min. After this, the mixture was centrifuged at 5000 g for 10 min and the aqueous layer was collected and the procedure repeated twice. Without standing for more than 30 min, extracts were analyzed for lipid oxidation.

MDA level in samples was quantified using the thiobarbituric acid (TBA) reactive substrates (TBARS) assay (De Leon & Borges, 2020). Each extract (2 mL) was added to trichloroacetitic acid (TCA) solution (4 mL 0.1 %), mixed and allowed to stand for 20 min at ambient temperature (~25 °C). TBA (2 mL 0.5 %) prepared in 20 % TCA was added to the reaction mixture (2 mL) and vortexed to mix. Sample-TCA mixture without TBA served as control. The reaction mixture was shaken in a hot water bath at 90 °C for 30 min to allow MDA-TBA complexation. The reaction was stopped by putting the tubes on ice to cool for 5 min after which the mixtures were centrifuged at 5000 g for 10 min. Supernatants (2 mL) were spectrophotometrically read using cuvettes at 532 nm and 600 nm. MDA level in samples was calculated according to Eq. 1.

$$\mu mol\ MDA g^{-1} = (2.\quad (m+v).\frac{\Delta Abs\ TBA - \quad \Delta Abs\ TCA}{m.b.E} \tag{1}$$

where ***m*** is mass in gram (g) of sample, ***v*** is the volume of extract used in the assay, ***b*** is optical path of the spectrophotometer cuvette, and ***E*** is the coefficient of extinction of MDA (155 mM^{-1} cm^{-1}). ΔAbs TBA = Abs_{532} nm TBA – Abs_{600} nm TBA and ΔAbs TCA = Abs_{532} nm TCA – Abs_{600} nm TCA.

The ferrous xylenol orange assay was used to determine the peroxides in treated samples (Ribourg-Birault & Genot, 2024). TTP extracts (1 mL) were mixed with chloroform: methanol (7:3) solution (9 mL) and xylenol orange (100 mL 10 mM) was immediately added. Fe^{2+} solution (50 mL 36 mM) was then added and the mixture was Vortexed for 5 s and allowed to stand for 5 min in the dark at 25 °C. The absorbance of reaction mixture was then read at 560 nm and PV were determined in meq O_2 kg^{-1} fat against a $FeCl_3$ calibration curve (R2 = 0.997).

2.2.5.3. Cellular antioxidant activity (CAA) determination. Using the Zen-Bio assay kit, the CAA was used to determine the capacity of phenolic extracts to inhibit dichlorodihydrofluorescein (DCFH) oxidation to its oxidized form, DCF by ROS produced in cells. DCF is fluorescent in cells and can be measured to determine antioxidant or pro-oxidant activity of substances. Extracts able to prevent the oxidation of DCFH to DCF are antioxidants and cells show reduction in fluorescence. However, extracts unable to prevent this phenomenon are pro-oxidants and cells show high fluorescence. The flavonoid, quercetin was used as a standard antioxidant.

HepG2 cells were cultured in RPMI and seeded in microtiter 96 wells plate until ~90 % confluence was achieved. Different concentrations of

quercetin solution and extracts were prepared (0 – 1000 μM). RPMI was removed and wells rinsed with PBS buffer once after which DCFH-DA probe solution (50 μL) was added to each well. Quercetin dilutions and extracts (50 μL) were added to designated cells containing DCFH-DA probe solution. Plate was incubated at 37 °C in 5 % CO_2 for 1 h, protected from light after which wells were rinsed with PBS (100 μL). Freshly prepared radical initiator (100 μL) was added to each well and the plate was read immediately over 60 min at 5 min intervals in a plate reader chamber preset at 37 °C at 485 nm and 538 nm, excitation and emission wavelengths, respectively. Control wells contained test samples, DCFH-DA and radical initiator without cells. The lack of DCF formation (i.e. no fluorescence) in control wells confirmed the absence of test-specific background errors from well contamination or test sample-reagent interactions. The area under the curve (AUC) was calculated using Eq. 2 (Pilaquinga et al., 2021).

$$AUC = 1 + \frac{RFU_1}{RFU_0} + \frac{RFU_2}{RFU_0} + \ldots \frac{RFU_{59}}{RFU_0} + \frac{RFU_{60}}{RFU_0} \quad (2)$$

RFU_0 = relative fluorescence unit at time point zero

RFUx = relative fluorescence unit at time points > 0

The AUC values were computed for both the control and the extracts and used to estimate CAA units according to Eq. 3.

$$CAA = 100 - \left[\left(\frac{AUC_{antioxidant}}{AUC_{control}}\right) \times 100\right] \quad (3)$$

2.2.6. Cytotoxic effect of TTP extracts on human liver cancer cells after 24 h treatment

It is believed that cytotoxic agents do not favour proliferation of cells as they induce cell death, hence the choice to assess the cytotoxicity of TTP extracts on the proliferation of human liver cancer cells (HepG2) according to the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay (Dzah, 2023). Briefly, $HepG_2$ cells were cultured, trypsinized, seeded into microtiter plate wells and incubated for 24 h at 37 °C in cell culture CO_2 incubator (Xvivo System Model X3, Biospherix, Parish, NY, USA). Extracts and quercetin standard (0–1000 μM) were added to each well and plates incubated (37 °C for 24 h). After removing extra test samples and rinsing with PBS buffer, MTT (20 μL of 5 mg mL^{-1}) was added to each well and incubated (37 °C for 4 h). DMSO (100 μL) was then added to each well to dissolve any formazan produced at room temperature (~30 °C) for 15 min in the dark. Absorbance was then read at 590 nm spectrophotometrically against control. Higher absorbance show higher cell viability whereas lower absorbance reflects the cytotoxic effect of treatments. Thus, the percentage viability and cytotoxicity of extracts were calculated according to Eq. 4 and Eq. 5, respectively.

$$\%Viability = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ control\ cells} \times 100 \quad (4)$$

$$\%Cytotoxocity = 100 - \%Viability \quad (5)$$

2.2.7. Identification and characterization of polyphenols

Lyophilized extract was reconstituted with deionized, distilled water to a concentration of 2.5 mg mL^{-1}, vortexed and passed through 0.22 μm filters for HPLC analysis. 1.5 mL of each filtrate was put into HPLC grade amber sample vials for analysis. HPLC was fitted with a ZORBAX C18 (80, 5 μm, 4.6 × 150 mm) column and a PDA-DAD detector set at a 150–600 nm wavelength range. Mobile phase solution A was made of distilled, deionized water (80 %) and 0.25 % acetic acid (20 %) and B contained 99 % acetonitrile and 1 % acetic acid. Using an injection volume of 10 μL, flow rate of 1 mL min^{-1} and at 40 °C column temperature, a gradient elution was used to analyze extracts (0 – 10 min, B: A = 5:95; 10 – 25, 15:85; 25 – 35, 30:70; 35 – 45, 50:50; 45 – 55, 70:30; 55 – 65, 85:15; 65 – 70, 90:10; and 70 – 75, 5:95). LC-MS analysis was also done, following HPLC analysis, using an Agilent LC instrument (Mod. 1100 Series, Agilent Technologies, Madrid, Spain) equipped with a G1376A binary capillary pump, a G1379A degasser and a G1315B diode array detector (500 nL, 10 mm pathlength) (150 – 600 nm). The capillary analytical column was a ZORBAX C18 (80, 5 μm, 4.6 × 150 mm). Agilent Chemstation software package for Microsoft Windows was used for data acquisition and processing. The instrument functioned in negative electrospray ionization (ESI) mode and N_2 was used to nebulize (1.0 L min^{-1}) and as a drying (10.0 L min^{-1}) gas. Argon was used as collision gas at a pressure of 230 kPa to induce collision dissociation in the collision cell and the collision energy voltages applied ranged from 10 to 55 eV. ESI voltage was set at −4.5 kV, the interface current was fixed at 6.2 μA, and the detector voltage at 1.84 kV. Data and peaks were analyzed and identified through NIST 17 library search by using the automated mass spectral deconvolution and identification system (AMDIS32) and the Thermo Scientific Xcalibur Qual browser. Data acquisition and processing were performed with the LabSolutions HPLC software.

2.2.8. Statistical analyses

Measurements for all experiments were done in triplicates and results were calculated as means with standard deviations. The IBM SPSS (version 26) and Microsoft Excel (Microsoft Office 2018) were used for data organization and analyses. Significant differences between treatments were determined at a 95 % confidence interval using analysis of variance and the Pearson correlation was used to determine the relationship between study variables. Results were presented in Tables and Figures.

3. Results and discussion

3.1. Total phenolic content determination

The TPC of extracts treated with $FeCl_3$ and ascorbic acid were 9.01 ±0.0.05 and 8.97 ±0.02 mg GAE g^{-1}, respectively, with no significant difference ($P > 0.05$), similar to estimates from previous studies conducted on TTP (Dzah, 2022, 2023). They were higher than TPC for TTP-HWE (5.30 ± 0.10 mg GAE g^{-1}), showing the superiority of ultrasound-assisted method to HWE in TPC recovery (Table 1). The high solvent-to-material ratio of 80 mL g^{-1} and the low acoustic frequency of 20 kHz was able to enhance the mass transfer of polyphenols into solution as observed in earlier studies (Dzah, 2022; Dzah, Duan, Zhang, Golly, et al., 2020).

Table 1
Antioxidant and pro-oxidant activities of $FeCl_3$- and ascorbic acid-treated TTP extracts.

TTP extract	TPC (mg GAE g^{-1} DW)	Antioxidant assay (% Inhibition)		Lipid oxidation test	
		DPPH	**ABTS**	**MDA (μmol g^{-1})**	**PV (meq O_2 kg^{-1} fat)**
TTP-$FeCl_3$	9.01 ± 0.05^{a}	11.00 ± 0.09^{a}	09.00 ± 0.10^{a}	2.53 ± 0.13^{a}	1.16 ± 0.07^{a}
TTP-Ascorbic acid	8.97 ± 0.02^{a}	72.00 ± 0.04^{b}	67.00 ± 0.06^{b}	0.15 ± 0.06^{b}	0.19 ± 0.03^{b}
TTP-Control (without additives)	9.18 ± 0.03^{a}	58.00 ± 0.07^{c}	58.00 ± 0.11^{c}	0.17 ± 0.03^{bc}	0.40 ± 0.00^{c}
TTP-HWE (without sonication)	5.30 ± 0.10^{b}	63.00 ± 0.08^{d}	64.00 ± 0.20^{d}	0.18 ± 0.01^{c}	0.20 ± 0.05^{b}

NB: Figures are reported as mean values ± standard deviation. Different letters in superscript along the same column depict significant difference at a $P < 0.05$ according to Duncan's Multiple Range Test.

3.2. Antioxidant activity and lipid oxidation assessment

3.2.1. DPPH and ABTS assays

The antioxidant properties of all extracts apart from TTP-$FeCl_3$ were significantly high, ranging from 58 % to 72 % and 58–67 % for DPPH and ABTS assays, respectively. TTP-$FeCl_3$ extract showed the lowest antioxidant activity of 11 % and 9 % for DPPH and ABTS, respectively (Table 1). There were significant differences between the antioxidant activity of all extracts with TTP-Ascorbic acid having the highest, followed by TTP-HWE, TTP-Control and TTP-$FeCl_3$. Ascorbic acid may have minimized ROS generation during UAE of polyphenols thereby conserving its antioxidant activity. TTP-Control which had no additive may have showed a lower antioxidant activity than TTP-Ascorbic acid due to ultrasound-induced ROS generation (Dzah, Duan, Zhang, Boateng, et al., 2020). The extremely low antioxidant activity of TTP-$FeCl_3$ extract was most likely due to enhanced ROS generation from the Fenton mechanism induced by Fe (III) in the presence of hydroxyl radicals and phenolic compounds. Quinones, semi-quinones and phenoxyl radicals with pro-oxidant properties may have been formed in TTP-$FeCl_3$ extract, accounting for its low antioxidant activity (Dzah et al., 2024).

3.2.2. Lipid oxidation determination by MDA and PV assays

Having noted form previous studies that Fe^{3+} ions can induce the production of ROS in the presence of polyphenols *via* the Fenton-like mechanism (Kruk et al., 2005; Segura et al., 2012), UAE was used enhance this process. Results have shown that TTP-$FeCl_3$ had the highest generation of MDA, followed by TTP-Control (without additive), TTP-Ascorbic acid and TTP-HWE (without sonication) (Fig. 4). At 60 min, TTP-$FeCl_3$ had 0.62 μmol of MDA per gram of sample, compared to TTP-Ascorbic acid (0.09 μmol g^{-1}), TTP-Control (0.18 μmol g^{-1}) and TTP-HWE (0.06 μmol g^{-1}) ($P < 0.05$). Ascorbic acid in TTP-Ascorbic acid may have acted as an antioxidant to scavenge ROS produced during UAE of TTP. This resulted in a reduction in MDA levels and PV of lipid. It also confirmed that the addition of $FeCl_3$ significantly produced ROS which was evident in the high MDA and PV observed for TTP-$FeCl_3$ (Table 1 and Fig. 4).

Moreover, without the addition of $FeCl_3$ and ascorbic acid (TTP-Control), UAE of polyphenols using water as a sole solvent generated ROS and induced lipid oxidation in a time-dependent manner (Fig. 4). Thus, the longer the extraction in water, the higher the generation of ROS and its induction of lipid oxidation, as confirmed by increased MDA levels and PV (Table 1) (Dzah, Duan, Zhang, Boateng, et al., 2020). Comparing a UAE (TTP-Control) with a non-UAE method (TTP-HWE), it was clear that the earlier favoured ROS generation and recorded significantly higher lipid oxidation (Table 1 and Fig. 4).

3.2.3. Cellular antioxidant activity determination

Liver cancer cells, like other cancer cells, generate high levels of ROS metabolically (Hayes et al., 2020; Nakamura & Takada, 2021). By design, they have adaptive mechanism to thrive under high oxidative stress (Arfin et al., 2021). Thus, the ability of TTP extracts to scavenge free radicals generated in liver cancer cells was investigated (Fig. 5 and Fig. 6). The AUC in Fig. 5 are indirectly related to the antioxidant potential of extracts. Thus, compared to the blanks (0 μM), increment in TTP extract concentrations for TTP-Control, TTP-HWE and TTP-ascorbic acid resulted in reduction in the area under the curves which indirectly confirmed increasing antioxidant activity. For TTP-$FeCl_3$, increased concentration of extract resulted in increased area under the curve, hence increased pro-oxidant activity. It means, compared to the other extracts, TTP-$FeCl_3$ possessed the least antioxidant potential (Fig. 5). This observation can be explained using the Fenton-like reaction principle that supports the generation of ROS in cells in the presence of transition metals such as Fe^{3+} (Abe et al., 2022). Although polyphenols have been known to inhibit the Fenton-induced production of ROS by complexing iron, this mechanism is dependent on the type of polyphenol and its ability to act as a multidentate ligand among other conditions (Pan et al., 2023). Also, the ratio of multidentate polyphenols to Fe^{3+} available may be crucial as a shortage in the earlier may still lead to ROS generation. This pro-oxidant property of TTP-$FeCl_3$ extract correlated positively with the significantly high levels of MDA it produced when exposed to lipids as well as its high PV (Table 1 and Fig. 5). The TTP-Control extract did not show as much antioxidant capacity as TTP-ascorbic acid and TTP-HWE extracts because the ultrasound treatment in water likely produced some free hydroxyl radicals which may have caused the degradation or oxidation of some polyphenols (Dzah, Duan, Zhang, Boateng, et al., 2020; Dzah & Dzigbor, 2023). The TTP-ascorbic acid extract contained ascorbic acid, a known antioxidant which protected polyphenols against ROS through its scavenging activity, hence its high antioxidant potential (Fig. 5 and Fig. 6), confirmed by the lowest PV and level of MDA generated (Table 1).

3.3. Cytotoxicity of TTP extracts in liver cancer cells

The ability of TTP extracts to kill human liver cancer cells was tested using to the MTT assay (Dzah, 2023). The different extracts exhibited different effects on the proliferation of the cancer cells (Fig. 7). All extracts were cytotoxic (i.e. significantly reduced cell viability), compared to their controls (0 μM) at all doses (Fig. 7). It was observed that extracts

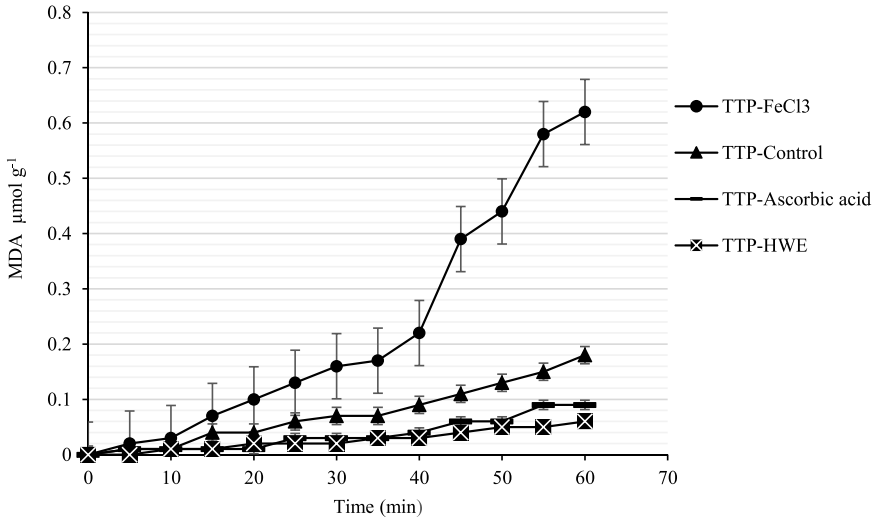

Fig. 4. Time-dependent influence of $FeCl_3$ and ascorbic acid on MDA levels in extracts.

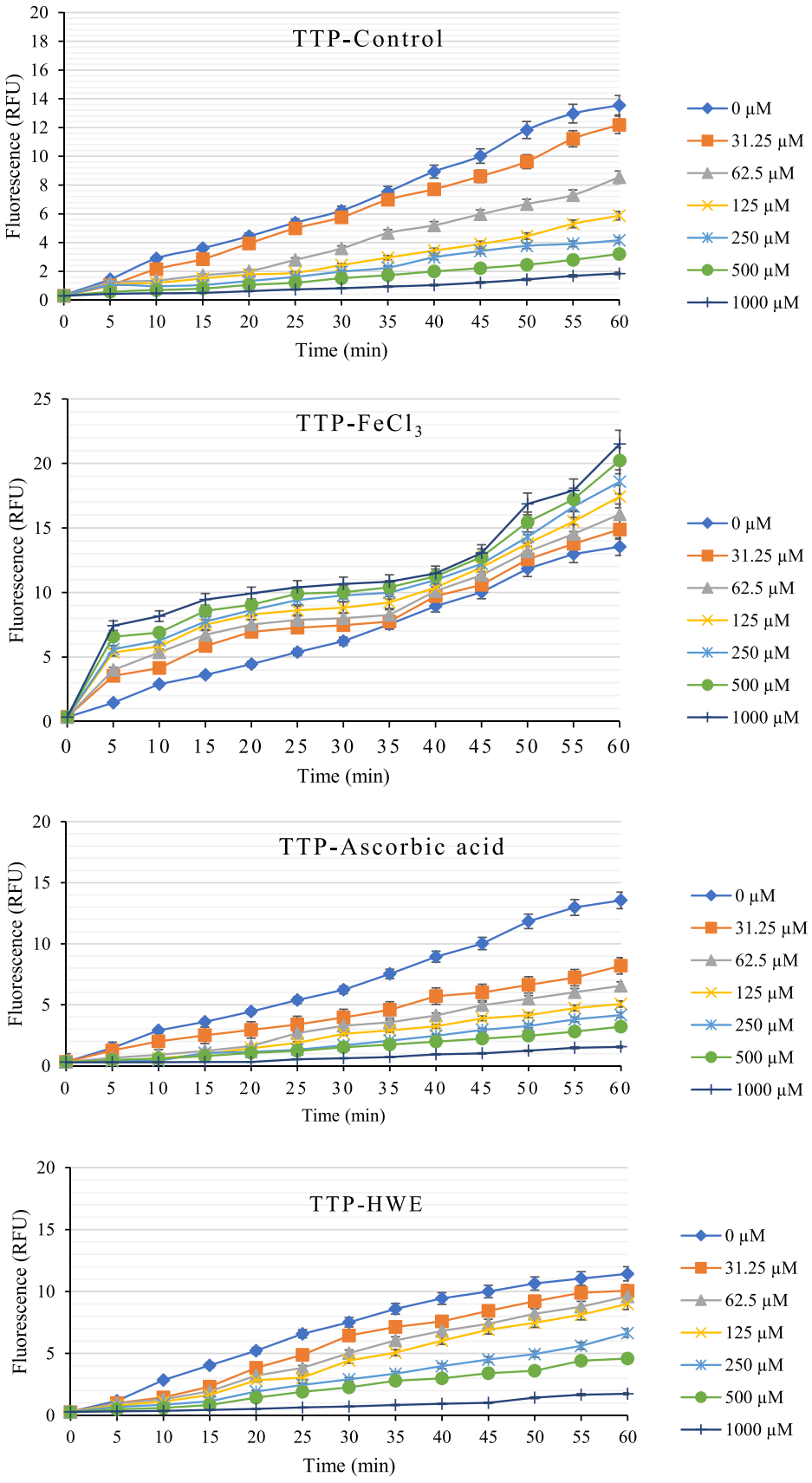

Fig. 5. Cellular antioxidant activity of *T. tetraptera* polyphenol (TTP) extracts by relative fluorescence units (RFU) in HepG2 cells.

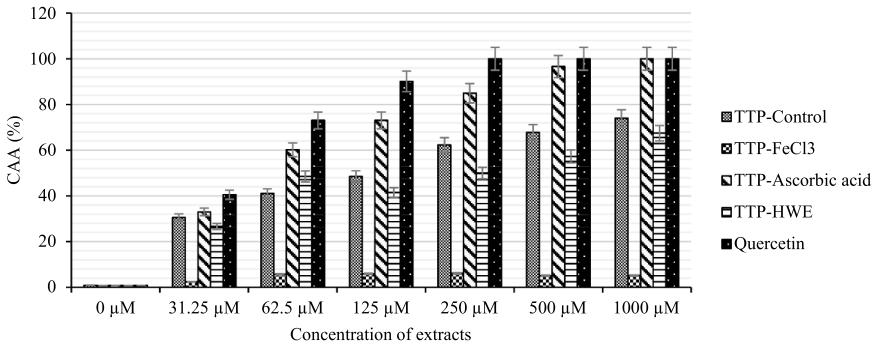

Fig. 6. Dose-dependent cellular antioxidant activity (CAA) of *T. tetraptera* polyphenol (TTP) extracts in HepG2 cells. Error bars depict standard error.

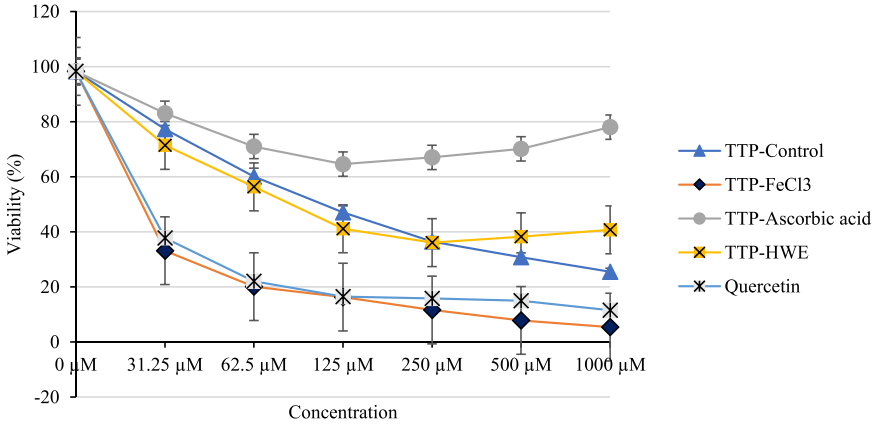

Fig. 7. Cytotoxic effects of different TTP extracts on liver cancer cells after 24 h treatment. Error bars depict standard error.

with high antioxidant property (i.e. TTP-Ascorbic acid > TTP-HWE > TTP-Control) showed a more gradual decrease in cell viability than TTP-$FeCl_3$ with low antioxidant property (Table 1 and Fig. 7). Apart from TTP-Control, TTP-Ascorbic acid and TTP-HWE treatments increased cell viability between 125 and 1000 µM. Between 125 and 1000 µM, TTP-Ascorbic acid increased cell viability from 64.59 % to 78.01 % after its initial decline from 98.27 % to 70.99 % between 0 and 62.5 µM dosage (Fig. 7). TTP-$FeCl_3$ with the least antioxidant property remarkably reduced cell viability from 98.27 % to 5.4 %, similar to quercetin (98.27–11.52 %) from 0 to 1000 µM. Quercetin showed high CAA but was able to induce cytotoxic effects in liver cancer cells (Fig. 6 and Fig. 7) as observed in many other studies (Hassan et al., 2020; Mohammed et al., 2021). The CAA of quercetin and its cytotoxic effect in liver cancer cells showed a positive correlation, whereas an inverse relationship was observed for all the other extracts with the exception of TPP-$FeCl_3$. On the other hand, the pro-oxidant activity of TTP-$FeCl_3$ in the cancer cells (Fig. 6) may have attributed to its ability to inhibit cancer cell viability in this study (Fig. 7).

In order to understand the pro-oxidant effect of TTP-$FeCl_3$ on liver cancer cell viability, it is essential to note that cancer cells have a characteristic high level of oxidative stress which they are able to adapt to due to their ability to produce glutathione (GSH) at levels that aid redox homeostasis (Dzah et al., 2024; Lu, 2013). However, beyond a certain threshold, increased oxidative stress from ROS causes redox-dependent apoptotic signaling through a cascade of mechanisms. Oxidative stress induces S-glutathionylation of GSH and its synthesis rate-limiting enzymes, hence inhibiting the GSH-dependent survival of cancer cells. Also, through ROS-induced proinflammatory cytokines, endothelial nitric oxide synthase (eNOS) which regulates the synthesis of nitric oxide (NO) is inhibited (Lu, 2013). This inhibits the translocation of glucose transporter 4 (GLUT4) to the cell membrane for glucose uptake and has the potential to induce caloric restriction in cancer cells, hence starving them to death (Dzah, Asante-Donyinah, et al., 2023; Dzah et al., 2024; Lu, 2013). All of the aforementioned mechanisms could have been induced by the pro-oxidant TTP-$FeCl_3$ extract which may have increased the oxidative stress of cancer cells beyond adaptable limits, resulting in its cytotoxicity (Fig. 7).

Quercetin's cytotoxic effect in cancer cells has been confirmed in a number of studies (Hassan et al., 2020; Mohammed et al., 2021). However, the mechanism underlying this may differ from that of TTP-$FeCl_3$. Several mechanisms have been proposed by different studies to explain the cytotoxic role of quercetin in liver cancer cells. According to some studies, quercetin has the ability to induce apoptosis by upregulating the Bax apoptotic regulator (Alam et al., 2022; Sethi et al., 2023; Wang et al., 2023). The cell death protease (caspase-3) and the cell cycle inhibitor gene (p21) were also upregulated by quercetin to induce apoptosis in liver cancer cells. It was also observed that while quercetin upregulated the expression of apoptotic genes, it downregulated those responsible for cell survival such as anti-apoptotic protein kinase B (PKB) and other cell cycle regulators such as PLK-1 and cyclins (B1 and A) which work with CDCs (CDC-2 and CDK-2) (Sethi et al., 2023). Many other studies have also reported quercetin's anticancer property to be mediated by its ability to disrupt the constitutive activation of the signal transducer and activator of transcription 3 (STAT3) protein necessary for cell proliferation and many other complex oncogenic mechanisms (Ghafouri-Fard et al., 2021; Yin et al., 2021).

In a previous study, TTP extracted in ethanol-water mixture (60:40, v/v) using a different ultrasound treatment (20 min, 20 kHz, 150 W, 50 °C, 26 mL g^{-1}) showed high cytotoxic effects on liver cancer cells especially at concentrations above 200 µg mL^{-1}, in a dose-dependent manner (Dzah, 2022). However, in this study, TTP extracted in water (TTP-Control) (45 min, 20 kHz, 300 W, 50 °C, 80 mL g^{-1}) rather showed proliferative activity in liver cancer cells in a dose-dependent manner (Fig. 7). It is therefore clear that differences in TTP extraction conditions

such as time, power, solvent-material ratio and solvent type may play significant roles in explaining the differences observed in the cytotoxic property of TTP. Thus, TTP extracts, depending on extraction conditions, may possess different chemical and bioactive properties.

3.4. Polyphenol identification and quantification by LC-ESI-MS

Compound characterization showed that eighteen compounds whose molecular weights ranged from 154.03 to 610.52 g mol^{-1} were identified in the TTP extract (Table 2, Fig. 8). Compared to an earlier study which extracted twenty-four (24) phenolic compounds from TTP, a significant number of these compounds were the same for both studies (Dzah, 2022). In this study, syringic acid, kaempferol 3,7-di-*O*-rhamnoside, isorhamnetin-3-*O*-glucoside, iso-orientin and epicatechin were the compounds that were not extracted in the earlier study. This may be due to the difference in extraction conditions in term of solvent, ultrasound power, extraction time and solvent-to-material ratio (Dzah, 2022). Also, phenolic compounds such as naringenin 7-*O*-glucoside, epigallocatechin, ellagic acid, galloyl glucose, ferulic acid 4-*O*-glucuronide, ferulic acid 4-*O*-glucoside, cyanidin 3-*O*-rutinoside, cyanidin-3-*O*-glucoside (chloride), and caffeic acid were not identified in this study unlike in the earlier conducted.

The major phenolic compounds extracted were kaempferol-3-*O*-rutinoside, 4-*O*-methylgallic acid, chlorogenic acid, rutin, quercetin-3-*O*-glucuronide, cyanidin-3-*O*-galactoside, orientin, isorhamnetin-3-*O*-glucoside and gallic acid (Table 2). The absorbances and concentrations of the phenolic compounds extracted were lower compared to earlier studies on pressurized hot water extraction (Dzah, 2023) and ultrasound-assisted ethanolic extraction (Dzah, 2022). Probably, the extended ultrasound extraction time (45 min) employed in this study may have resulted in significant degradation of polyphenols unlike in previous studies in which extraction was done for 20 min (Dzah, 2022). Also, due to the excessive use of water as the sole solvent in this study, free hydroxyl radicals, combined with high microenvironmental temperature, may have resulted in phenolic degradation as well (Bremner et al., 2006).

3.5. Relationship between antioxidant potential of extracts and their cytotoxicity in HepG2 cells

To assess the relationship between antioxidant and pro-oxidant properties and cytotoxicity of TTP extracts at 1000 μM dosage, data were correlated using the Pearson correlation (PC) matrix in Microsoft excel (version 21) (Table 3). Due to the inverse relationship between the antioxidant property of quercetin and its cytotoxic effect which deviated from that of other TTP extracts, it was excluded from the PC analysis.

Higher antioxidant activity of TTP extracts determined according to the DPPH, ABTS and CAA assays generally correlated negatively with lipid oxidation products, MDA and PV with coefficients ranging between −0.94 and −1.00 (Table 3). This infers an opposite relationship between the two sets of variables, meaning that the higher the antioxidant property of extracts, the lower the level of MDA and PV (Table 1). Thus, antioxidant compounds in TTP extracts inhibited the oxidation of lipids as confirmed in similar studies (Dzah, Duan, Zhang, Boateng, et al., 2020; Feng et al., 2010). This explains why TTP-$FeCl_3$ extract with the lowest antioxidant activity produced the highest levels of oxidation products (Table 1). Concerning cytotoxicity, higher antioxidant activity of TTP extracts resulted in higher viability of HepG2 cells (i.e. lower toxicity) with correlation coefficients being 0.87, 0.83 and 0.78 for CAA, DPPH and ABTS, respectively (Table 3). This is also in line with the cytotoxicity of extracts shown in Fig. 7, with TTP-$FeCl_3$ showing the highest cytotoxicity although it had the least antioxidant potential. MDA and PV which indirectly show low antioxidant potential of extracts negatively correlated with HepG2 cell viability (-0.70 and −0.79,

Table 2
Identification of phenolic compounds in *T. tetraptera* polyphenols (TTP) extracted in water using ultrasound.

No	Compound	Molecular formula	Retention time (min)	Mode of Ionization	Molecular weight (g/mol)	Theoretical (*m/z*)	Observed (*m/z*)	Fragmentation ions	Concentration (μg/g)
	Hydroxybenzoic acids								
1	Gallic acid	$C_7H_6O_5$	4.94	$[M-H]^-$	170.12	169.12	169.12	125, 109, 85, 71, 43	**10.47**
2	2,3-Dihydroxybenzoic acid	$C_7H_6O_4$	12.10	$[M-H]^-$	154.03	153.03	153.01	138, 123, 109, 85, 43	8.06
3	4-*O*-Methylgallic acid	$C_8H_8O_5$	14.07	$[M-H]^-$	184.15	183.14	183.15	165, 151, 135, 121, 97	**19.05**
4	Syringic acid	$C_9H_{10}O_5$	39.04	$[M+H]^+$	198.18	199.17	199.18	179, 165, 151, 135, 121	7.13
	Hydroxycinnamic acids								
5	p-Coumaric acid 4-*O*-glucoside	$C_{15}H_{18}O_8$	18.43	$[M-H]^-$	326.30	325.29	325.29	303, 285, 265, 247, 175	7.38
6	Chlorogenic acid	$C_{16}H_{18}O_9$	20.90	$[M-H]^-$	354.31	353.14	353.14	191, 179, 161, 135, 89	**16.22**
	Flavonoids								
7	Rutin	$C_{27}H_{30}O_{16}$	5.32	$[M-H]^-$	610.52	609.14	609.13	447, 303, 285, 273, 151	**16.15**
8	Isorhamnetin−3-*O*-glucoside	$C_{22}H_{22}O_{12}$	6.41	$[M-H]^-$	478.40	477.40	477.42	315, 293, 285, 271, 151	**10.11**
9	Kaempferol 3,7-di-*O*-rhamnoside	$C_{27}H_{30}O_{14}$	8.63	$[M+H]^+$	578.52	579.52	579.51	577, 449, 303, 285, 151	7.70
10	Kaempferol−3-*O*-rutinoside	$C_{27}H_{30}O_{15}$	8.50	$[M-H]^-$	594.52	593.52	593.54	463, 303, 285, 151	**23.75**
11	Quercetin−3-*O*-glucuronide	$C_{21}H_{18}O_{13}$	9.93	$[M+H]^+$	478.36	479.36	479.36	315, 293, 285, 151	**13.19**
12	Catechin	$C_{15}H_{14}O_6$	10.94	$[M-H]^-$	290.28	289.27	289.27	245, 227, 205, 151	9.44
13	Cyanidin−3-*O*-galactoside	$C_{21}H_{21}O_{11}$	17.65	$[M+H]^+$	484.84	485.10	485.10	301, 287, 151	**12.58**
14	Orientin	$C_{21}H_{20}O_{11}$	27.23	$[M-H]^-$	448.38	447.38	447.36	447, 303, 285, 151	**10.99**
15	Epicatechin	$C_{15}H_{14}O_6$	27.96	$[M+H]^+$	290.27	291.27	291.28	289, 245, 227, 205, 151	4.07
16	Iso-orientin	$C_{21}H_{20}O_{11}$	28.05	$[M+H]^+$	448.38	449.38	449.37	447, 303, 285, 151	3.06
17	p-Coumaric acid	$C_9H_8O_3$	28.76	$[M+H]^+$	164.16	165.05	165.04	163, 119, 105, 91	1.94

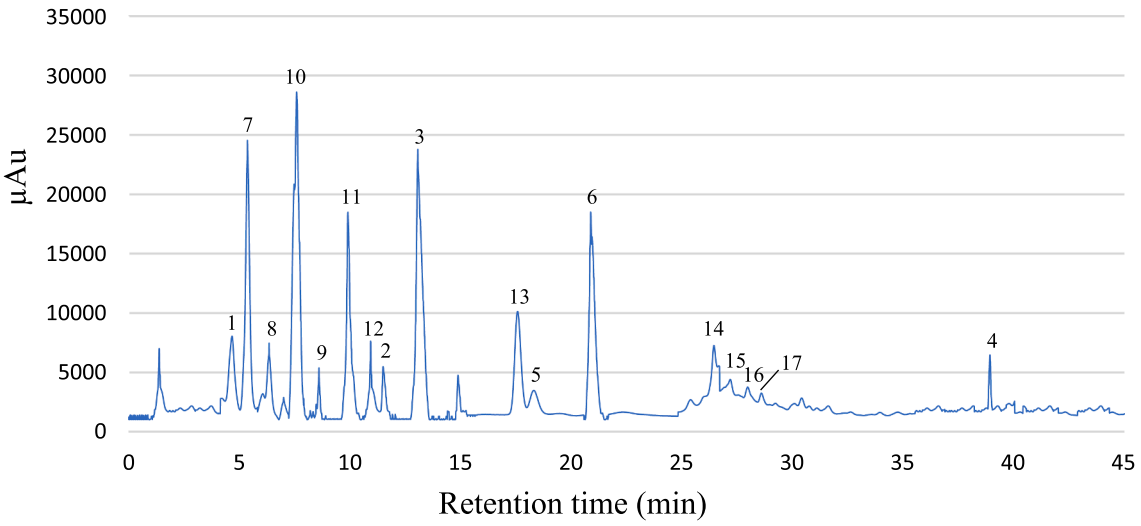

Fig. 8. LC-ESI-MS chromatogram of *T. tetraptera* polyphenol extracts.

Table 3
Pearson correlation showing the relationship between variables.

	MDA	*PV*	*CAA*	*DPPH*	*ABTS*	*CYT (% proliferation)*
MDA	1					
PV	0.977968	1				
CAA	−0.94036	−0.94267	1			
DPPH	−0.97888	−0.99101	0.97868	1		
ABTS	−0.9912	−0.99599	0.957555	0.995295	1	
CYT (% proliferation)	−0.70149	−0.7937	0.874818	0.831632	0.778505	1

respectively) (Table 3 and Fig. 7). This effect was induced by TTP-$FeCl_3$ due to its low antioxidant activity (Table 1 and Fig. 7).

4. Conclusion

This study explored the potential use of ultrasound and the Fenton-reaction to produce pro-oxidant polyphenol extracts from TT. Some of these extracts have been tested for their biological properties and they have exhibited pro-oxidant characteristics evident in their enhancement of lipid oxidation product MDA production and their poor ROS inhibition as observed using the DPPH and ABTS assays. Their effects on the proliferation of liver cancer cells were promising, in that they inhibited proliferation by inducing cytotoxicity. Compared to the quercetin standard which is well-known as a hepatotoxic agent, TTP-$FeCl_3$ extract performed favorably, inducing cytotoxicity in a similar but mechanistically different manner.

The purified TTP-$FeCl_3$ extract contained seventeen phenolic compounds with low absorbances and low concentrations. Thus, the treatment, coupled with $FeCl_3$-induced oxidation, was suspected to have induced polyphenol oxidative degradation, hence the pro-oxidant and anti-proliferative properties observed. The use of ascorbic acid to minimize oxidative degradation in TTP-Ascorbic acid resulted in an extract with high antioxidant activity.

This study forms a basis to use prolonged high-power, low-frequency ultrasound treatment and Fe^{3+} to produce pro-oxidant polyphenols capable of fighting liver cancer cells *in vitro*. Similar approach can be used to degrade phenol pollutants through ultrasound-induced generation of free hydroxyl radicals. Future studies may consider the influence of pH on the outcome of $FeCl_3$ treatment of TTP extracts regarding their anti-/ pro-oxidant potentials and other biological activities.

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CRediT authorship contribution statement

Courage Sedem Dzah: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Constance Agbemelo-Tsomafo:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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