



Flavonoids and phenols of *Sclerocarya birrea* fruit exocarp extracts differentially modulating antioxidant activities

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Received: 17 August 2023 / Accepted: 21 August 2025 / Published online: 18 September 2025
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Abstract

The aim of this study was to evaluate the total content of flavonoids and phenols, in vitro antioxidant and cytoprotective effects of *Sclerocarya birrea* fruit exocarp extracts. The fruit exocarp was extracted sequentially in Hexane, Chloroform, Ethyl acetate, and Methanol and the antioxidant properties were evaluated using in vitro antioxidant assays and cytoprotective effects against H₂O₂-induced cell death using HeLa cells. Molecular docking using Autodock 4.2.6 was used to assess the binding interactions of *Sclerocarya birrea* compounds to the antioxidant enzymes. The total flavonoid and total phenolic contents were in the order Methanol>Ethyl acetate>Chloroform>Hexane ($P<0.05$) of *Sclerocarya birrea* fruit exocarp extracts. The antioxidant properties using DPPH, ABTS, and Ferric reducing antioxidant power, and total antioxidant capacity assays revealed significantly higher ($P<0.05$) antioxidant activity in the Methanol>Ethyl acetate>Chloroform>Hexane. Using WST-1 assay, H₂O₂-induced cell loss was effectively reduced by preincubating HeLa cells with Ethyl acetate>Methanol>Chloroform>Hexane ($P<0.05$) extracts. Methanol and Ethyl acetate extracts also significantly increased ($P<0.05$) the activities of Superoxide dismutase, Catalase and reduced Glutathione. Amongst the identified compounds, Myricetin 3-o- α - rhamnoside (C8) emerged as the best compound to bind to both SOD and CAT with binding energies of -9.1 and -8.8 kcal/mol respectively. C8 formed hydrogen bonds and electrostatic interactions with SOD and CAT to act as an agonist. Our findings suggest that Methanol and Ethyl acetate *Sclerocarya birrea* fruit exocarp extracts improved antioxidant capacity and thus have the potential to be developed into antioxidant products that will alleviate oxidative stress linked diseases.

Keywords *Sclerocarya birrea* · ROS · Antioxidants enzymes · Cytoprotective · Molecular docking

Introduction

Oxidative stress is a toxic physiological environment that is a precursor for several metabolic and degenerative diseases (Jelic et al. 2021; Darenskaya et al. 2021). This environment is characterized by a significantly higher concentration of Reactive oxygen species (ROS) than cells require (Incalza et al. 2018). Reactive oxygen species (ROS) are highly unstable, short lived and reactive oxygen-derived radicals that rapidly oxidize nucleic acids, lipids and proteins within their vicinity to attain molecular stability (Ozougwu 2016). The reactivity of ROS is due to their chemical composition, they have an uneven distribution of electrons resulting in partially negative charged molecule. ROS are generated from by cells during metabolism as they are terminal electron acceptors in several signalling pathways such as inflammation, oxidative phosphorylation in the

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mitochondrial membrane, endoplasmic reticulum and other cellular pathways (Bhattacharjee 2019). Antioxidants are chemical entities with the ability to reduce ROS either by electron or hydrogen transfer, as well as through chemical bonding (Gulcin 2020). There are those coded by the body, commonly referred to as Endogenous enzymatic antioxidants such as Superoxide dismutase (SOD) (Gottfredsen et al. 2013), and Catalase (CAT) (García-Molina et al. 2005). The body also produces non-enzymatic endogenous antioxidants such as Glutathione (GSH), which is affected during oxidation resulting in an inactive form known as glutathione disulfide (GSSH) (Armogida et al. 2012). Exogenous antioxidants from plants have been used as supplements to reinstate the antioxidant properties of GSSH to GSH as well as directly neutralizing ROS (Mironczuk-Chodakowska et al. 2018).

Sclerocarya birrea is a savannah tree that belongs to a family of Anacardiaceae grown in sub-Saharan Africa. Studies on *Sclerocarya birrea* stem barks, leaves, and roots extracts have reported the presence of groups of compounds antioxidants capacities such as polyphenols, flavonoids, saponins and tannins (Manzo et al. 2017; Russo et al. 2018; Virginie et al. 2016). Research has also indicated several biological activities such antidiarrheal, antimicrobial, antiplasmodial, anti-inflammatory, antidiabetic, anti-hypertension, and antiseptic properties from the bark and leaves extracts of *Sclerocarya birrea* (Ojewole et al. 2010). However, there are limited studies on the fruit exocarp. The exocarp may have been overlooked because it is considered as waste by most of consumers, it is not part of the diet. However, *Sclerocarya birrea* fruit exocarp may be a convenient and rich source of phytochemicals vital to our body. Therefore, this study evaluated the total content of flavonoids, and phenols in the extracts of the fruit exocarp, as well as the in vitro antioxidant activities in order to establish if it's a candidate for organic antioxidants that can be developed into supplements or drugs with clinical implementations. This study presents an opportunity to assess if the fruit exocarp can be incorporated as an alternative management approach for oxidative stress-based diseases such as cancer and diabetes.

Methology

Plant collection and authentication

Sclerocarya birrea fruit samples were collected at coordinates '22°35'35.7" S 27° 07' 28.5" E', which is in the township of Palapye, located in the Central district of Botswana. The plant was authenticated at the Herbarium laboratory at University of Botswana, voucher number BAK-02/2020.

Sample preparation

Sclerocarya birrea fruits were pilled to separate the exocarp from the mesocarp after being rinsed with distilled water. The fruit exocarp was cleaned with distilled water before being dried for 48 h in a Lasec MMMERT UF 110 laboratory oven at 40 °C. Using a Salton SB400E blender, the dried materials were crushed into a powder before being sequentially extracted in Hexane, Chloroform, Ethyl acetate, and Methanol as prescribed by Mustafa et al., (2020) with minor changes. In round bottom flasks, precisely 300 g of dry powder were soaked in 1 L of hexane for 76 h before being filtered through Whatman filter paper, Grade 1 (Merck, South Africa). Following a 6-h air drying period, the plant residue was once more immersed in 1 L of chloroform for 76 h. The procedure was repeated using ethyl acetate and methanol.

Determination of total flavonoid content (TFC)

The aluminum chloride colorimetric assay was used to calculate the TFC of the fruit exocarp of *Sclerocarya birrea* as described by Sembiring et al. (2018). In separate tubes, an aliquot (0.5 mL) of the extract and standard solution of quercetin (0.0312, 0.0625, 0.125, 0.25, 0.5, 1 mg/mL) was obtained. 2 mL of distilled water and 0.15 mL of sodium nitrite (5% NaNO₂, w/v) were then added, and the mixture was allowed to stand for 6 min. After incubating for 6 min, 0.15 mL aluminum chloride (10% AlCl₃) and 2 mL sodium hydroxide (4% w/v, NaOH) were added, and the volume was then increased to 5 mL with distilled water. The liquid turns pink after 30 min of incubation, and a Thermo Scientific TM SPECTRONICTM 200 Spectrophotometer was used to determine its absorbance at 420 nm. Distilled water was used as a blank. Every analysis was done in triplicates, represented as mg quercetin equivalent (QE)/g material, and the TFC was calculated as

$$\text{TFC} : \frac{cV}{m}$$

where *c*=concentration of extract equivalence to that of quercetin (quercetin standard curve), *V*=volume of extracts in mL and *m*=mass of extracts in grams.

Determination of total phenolic content (TPC)

Sclerocarya birrea's TPC was calculated utilizing a technique used by (Singleton et al. 1999). 200 µL of 10% (v/v) Folin-Ciocalteu reagent, 2 mL of distilled water, and an aliquot (0.2 mL) of the extract or standard solution of gallic acid (0.0312, 0.0625, 0.125, 0.25, 0.5, 1 mg/mL) were mixed and incubated for 8 min in the dark. 600 µL

of 20% (w/v) sodium carbonate was then added, and the mixture was incubated for 2 h at room temperature in the dark. Every analysis was done in triplicates. Thermofisher Scientific, USA's Spec200E UV-V spectrophotometer was used to measure the amount of absorbance at 760 nm. The data for gallic acid was used to plot a standard curve ($y=0.12+0.37x$, $R^2=0.99$) that was used in the analysis.

$$\text{TPC} = \frac{cV}{m}$$

where c =concentration of extract equivalence to that of gallic acid (from gallic acid standard curve), V =volume of extracts in mL and m =mass of extracts in grams.

Antioxidants assays

DPPH assay

The method employed to ascertain the radical scavenging activity of extracts on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was modified, as described by (Lv et al. 2018). Gallic acid (1 mg/mL) and extracts (1 mg/mL) were serially diluted to achieve concentrations of 0.0312, 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL. The wells of a 96-well plate received 150 μ L of 0.1 M DPPH. 50 μ L of plant extracts were used to treat DPPH for wells marked as treatment. A 50 μ L solution of gallic acid was used to treat the DPPH in the wells selected as positive controls. DPPH was given a 50 μ L methanol treatment for the wells designated as a negative control. At room temperature and in the dark, the reactions were incubated for 30 min. After 30 min, optical density (OD) was measured at 518 nm using a MULTISKAN FC microplate reader from Thermo Scientific (USA). The following is how the percentage inhibition (decolorization) was calculated:

$$\text{DPPH inhibition(\%)} : \frac{(\text{OD sample} - \text{OD control})}{\text{OD control}} \times 100$$

FRAP assay

With a few changes, Berker et al. (2007)'s description of this Ferric Reducing Antioxidants Power (FRAP) was employed. 8.3 mL of hydrochloric acid (HCL) and 100 mL of distilled water were mixed to create 1.0 N HCL. Gallic acid and plant extracts were serially diluted into quantities of 0.0312, 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL. A 96-well plate had 40 μ L of each concentration of plant extracts, methanol only and gallic acid added to the wells. The 96 well plates received 100 μ L of 1.0 N HCL. 20 μ L of sodium dodecyl sulphate at 1% and 30 mL of potassium

ferrocyanide at 1% came next. The reaction was allowed to sit at 50 °C for 20 min. Wells treated with gallic acid designated positive control while wells treated with methanol were used as negative control. Using a MULTISKAN FC microplate reader from Thermo Scientific (USA), absorbance was measured at 750 nm.

$$\text{FRAP reduction(\%)} : \frac{(\text{OD control} - \text{OD sample})}{\text{OD control}} \times 100$$

ABTS assay

The 2,2'-azino-bis (3-ethylbenzothiazoline6-sulfonic acid (ABTS) approach was applied in accordance with Kooy et al. (2016)'s instructions. This technique is based on ABTS radical cation decolorization. To make the ABTS working solution, 100 mL of 7 mM ABTS and 100 mL of 2.4 mM potassium persulfate were combined (1:1) and left at room temperature for 16 h in the dark. 1 mL of ABTS solution was gradually diluted with methanol until an absorbance of 0.706 ± 0.01 was attained at 734 nm. Then, extracts and gallic acid concentrations of 0.0312, 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL were added, and methanol was added to wells used as negative control, followed by 1 mL of the ABTS solution. The reaction was mixed and then allowed to sit for 7 min at room temperature with no light. Thermo Scientific's MULTISKAN FC microplate reader (available in the United States) was used to detect absorbance at 734 nm. The standard was gallic acid (positive control). This is how percentage inhibition was calculated:

$$\text{ABTS inhibition(\%)} : \frac{(\text{OD control} - \text{OD sample})}{\text{OD control}} \times 100$$

Total antioxidants capacity (TAC) using phosphomolybdenum

TAC is an assay that is based on the formation of a stable green phosphate-Mo(V) complex as an outcome of the electron reduction of Mo (VI) to Mo(V), as stated by (Sharadanand Phatak et al. 2014). 1 mL of phosphomolybdenum (made by combining 31.4 mL of 0.6 M H₂SO₄, 397.49 mg of 28 mM sodium phosphate, and 494.36 mg of 4 mM ammonium molybdate) was mixed with around 0.1 mL of extracts and gallic acid. In an IncoCool LABOTEC incubator (LAMWORLD TECHNOLOGIES PTY LTD), the mixture was heated to 95 °C for 90 min before being cooled to room temperature. Absorbance was measured at 950 nm using a Spec200E UV-V spectrophotometer (Thermofisher Scientific, USA). Gallic acid was used to make a standard ($y=0.12+0.39x$, $R^2=0.97$), from which TAC of extracts was interpolated.

Cell culture studies

Preparation of plant extracts concentrations

A stock solution of 10 mg/mL of plant extracts was obtained by dissolving 100 mg of the extracts in 10 mL of 10% (v/v) dimethyl-sulfoxide (DMSO). To achieve a concentration of 400 µg/mL, 10 mL of stock solution was added to 25 mL of Dulbecco's minimum essential medium (DMEM). 400 µg/mL has been adopted from Sharadanand Phatak et al. (2014). Serial dilutions of the 400 µg/mL resulted in concentrations of 12.5, 25, 50, 100, 200, and 400 µg/mL.

Cell culture

To conduct multiple studies on the cytoprotective properties of *Sclerocarya birrea* fruit exocarp extracts, HeLa cells were grown. Penicillin–streptomycin (10 000 U/mL: 10 000 g/mL) and 10% of fetal bovine serum (FBS) were added to high glucose DMEM, which was used to culture HeLa cells. At 37 °C and 5% CO₂, cells were incubated in a Thermo Scientific Forma SERIES 11 Water Jacket CO₂ Incubator. Before being seeded into flat bottom 96-well plates, cells were counted using a Countess TM 3 Automated Cell Counter. Cell concentrations were then generated according to the output of the cell counter to achieve 1×10^5 /mL.

Effects of extracts on oxidative stress biomarkers

A concentration of 1×10^5 /mL of 80–90% confluent HeLa cells (day 6 of passage of number 5) was treated for 2 h with 100 µL of the extracts (400 µg/mL), with untreated cells serving as the experiment's control. Using trypsin, cells were detached, then centrifuged at 1000 g for 10 min. The pellet was stirred up in cold PBS before being centrifuged at 1000 g for 10 min. The pellet was frozen for roughly 3 h at –20 °C after being suspended in cold PBS. The pellet was frozen, thawed, frozen again, and thawed again 3 times to shatter the cells. For further investigation, the cell homogenate was kept in ice after being centrifuged at 1500 g for 10 min. Utilizing ELISA kits (Elabscience, USA) in accordance with the manufacturer's instructions, the effects of extracts on the activities of superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) were assessed.

Cytoprotective effect of extracts against hydrogen peroxide-induced cell death using WST-1 assay

At day 6 of passage number 5, about 90–98% of confluent HeLa cells were trypsinized, seeded in 96-well plates at a density of 1×10^5 /mL, and cultured for 72 h. For the

wells designated as treatment, media was aspirated after incubation and replaced with 100 µL of *Sclerocarya birrea* fruit exocarp extracts (12.5, 25, 50, 100, 200, and 400 µg/mL). Cells in the positive control wells received PBS only. Cells were incubated for 2 h following treatment with plant extracts. By adding 10 µL of 1 mM hydrogen peroxide (H₂O₂) to the wells containing extracts and wells with cells treated with PBS only (positive control), and incubating for another 2 h, cells were exposed to hydrogen peroxide-induced oxidative stress. The 1 mM H₂O₂ was adopted from Mezghani et al. (2015). Cells were not given any treatment in negative control wells. 10 µL of (4-[-3(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate) WST-1 was added to the wells after the incubation period and incubated for an additional 3 h. Thermo Scientific's MULTISKAN FC microplate reader (480 nm wavelength) was used to measure the absorbance. To adjust the absorbance of the treatment and control wells, background absorbance from wells containing medium and 10 µL of WST-1 was taken into account. The number of live cells that were preserved was computed as a percentage and compared to the absorbance of the control group.

$$\text{Viable cells (\%)} : \frac{(\text{OD negative control} - \text{OD treated cells})}{(\text{OD negative control})} \times 100$$

In silico studies

Retrieval and preparation of ligands

Phytochemicals of *Sclerocarya birrea* were obtained from Russo et al. (2013) and downloaded from PubChem (www.pubchem.com) as 3D structures in SDF format and were converted to PDB format using BIOVIA Discovery Studio. The Autodock tools 1.5.6 was used to add Gasteiger charges and polar hydrogens and saving each compound in pdbqt format.

Retrieval and preparation of target proteins

The 3D structure of Superoxide dismutase (PDB ID: 1CB4) and catalase (PDB ID: 2CAG) was downloaded from the Protein Data Bank (www.pdb.com). Each protein was cleaned in Autodock tools by removing water molecules, and bound ligand, and prepared by adding Kollman charges and polar hydrogens, then saved in pdbqt file.

Molecular docking

Molecular docking was performed using the Autodock 4.2 program. Briefly, the grid parameter file was prepared using Autogrid 4 in Autodock tools and grid box set at as x = 10.41,

$y=87.88$, $z=18.62$ for SOD, and $x=58.38$, $y=19.08$, $z=18.3$ for CAT to cover the entire catalytic sites (Rana et al. 2019). The grid box dimensions were $40 \times 40 \times 40$ with a spacing of 0.375 angstroms. Then the docking parameter file was prepared using Autodock 4 in Autodock tools utilizing the genetic algorithm with 2,500,000 energy evaluations as the search parameter and docking using Lamarckian genetic algorithms utilizing Cygwin. Docked complexes (protein+ligand) were analyzed for their interactions using BIOVIA Discovery Studio Visualizer.

Data analysis

A Graph prism software was used to analyze data and develop graphs, generated results are presented as mean $\pm$ standard error of triplicates ($n=3$). To compare variation between the means at 95% confidence level ($p<0.05$), we used a One way- analysis of variance (ANOVA) with Tukey–Kramer's range test.

Results

Total Flavonoid and phenolic contents

In quantitative phytochemical screening of total flavonoid content (TFC) and total phenolic content (TPC) of extracts, Fig. 1A shows that methanol extracts showed significantly higher ($P<0.05$) TFC of 55.2 ± 3.8 mg QE/g. The Ethyl acetate extracts recorded the 2nd highest TFC with

47.7 ± 2.9 mg QE/g, while Chloroform extracts recorded the 3rd highest with 27.7 ± 5.0 mg QE/g. with Hexane showing the lowest TFC value of 20.2 ± 5.3 mg QE/g. Figure 1B reveals that Methanol extracts have a significantly higher ($P<0.05$) TPC of 105.4 ± 1.4 mg GAE/g, followed by Ethyl acetate (84.3 ± 2.6 mg GAE/g), Chloroform (67.8 ± 1.12 mg GAE/g), and Hexane showing the lowest TPC value of 42.3 ± 5.4 mg GAE/g.

Antioxidants assays

To evaluate radical neutralizing ability of extracts, we employed DPPH, ABTS, FRAP assays in serial dilutions while TAC assay was carried out at the highest concentration of 1 mg/mL. Generally, we observed a direct proportionality between radical scavenging, extract polarity, and extract concentration in serial diluted assays. We also observed a direction proportionality between TAC value's and extract polarity. As shown in Fig. 2A–C, methanol extracts recorded the highest inhibitions of $71.44 \pm 0.44\%$, $94.89 \pm 0.03\%$, and $82.75 \pm 3.48\%$ at 1 mg/mL for DPPH, FRAP and ABTS respectively. However, these values were lower than that of gallic acid (standard), which recorded $86.83 \pm 0.17\%$, $95.1 \pm 0.04\%$ and $86.72 \pm 2.19\%$ for the same assays. Methanol extracts were then followed by Ethyl acetate extracts, Chloroform extracts and Hexane extracts across DPPH, FRAP, and ABTS. Ethyl acetate extracts recorded the 2nd highest inhibitions with $55.73 \pm 0.25\%$, $91.19 \pm 0.10\%$, $59.01 \pm 0.84\%$ for DPPH, FRAP and ABTS respectively. Chloroform extracts recorded the 3rd

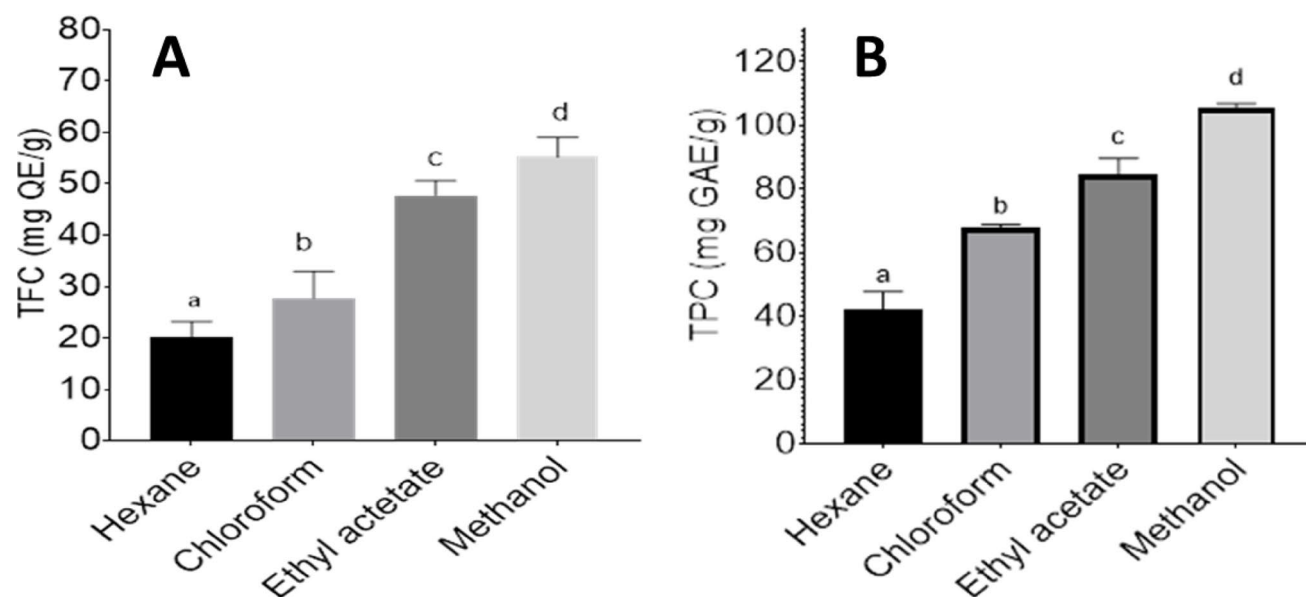


Fig. 1 Total flavonoid content (A) and total phenolic content (B) of the extracts of *Sclerocarya birrea* fruit exocarp, expressed in mg of quercetin equivalence per 1 gram of dry mass of an extract (mg QE/g) and mg of gallic acid equivalence per 1 gram of dry mass of an extract

(mg GAE/g) respectively. Data presented as mean $\pm$ standard error ($n=3$). Significant differences ($p<0.05$) are indicated by letters above the bars, where different letters depict significant differences between average means

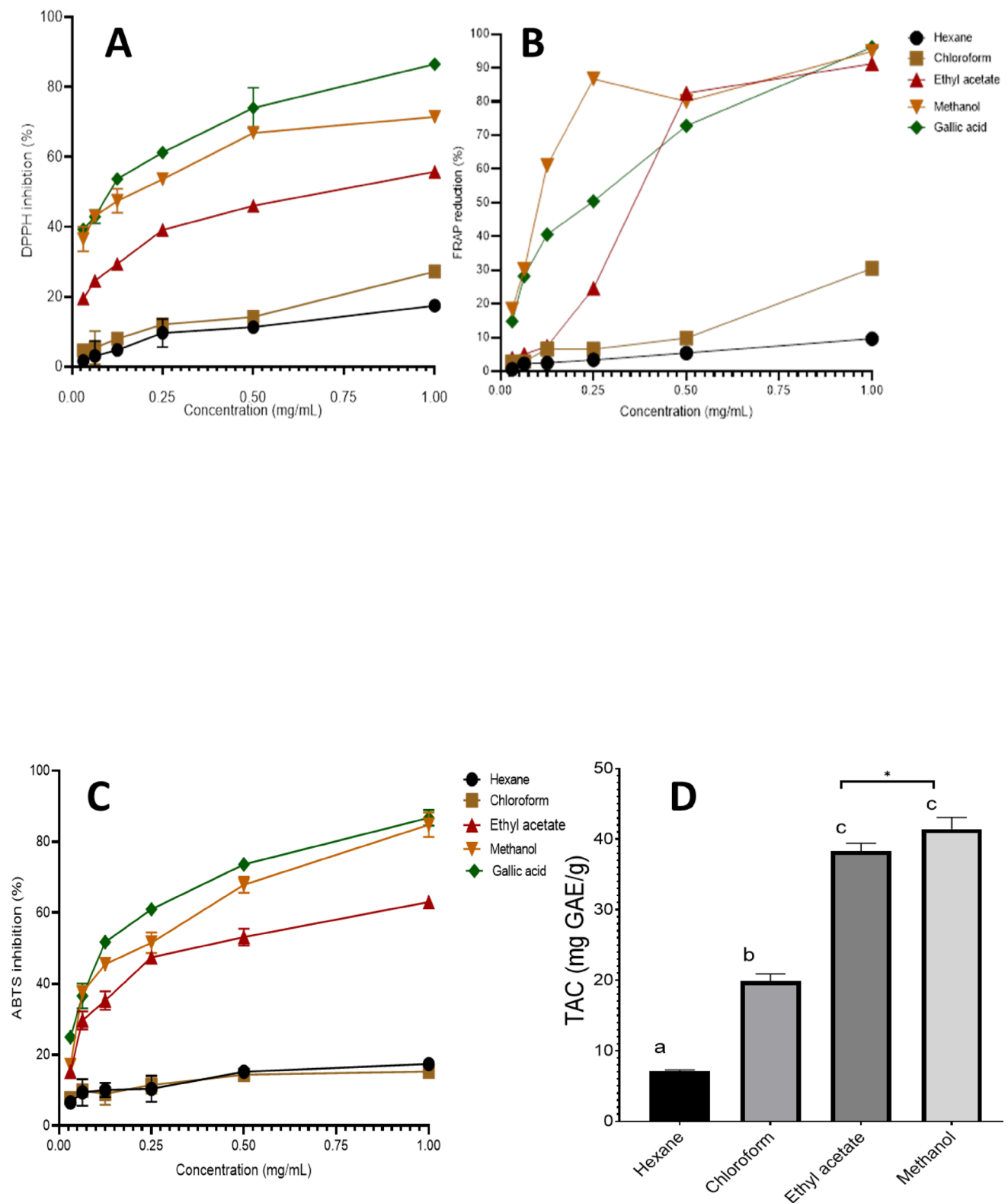


Fig. 2 The antioxidant effects of *Sclerocarya birrea* fruit exocarp extracts evaluated by **A** DPPH, **B** FRAP, **C** ABTS and **D** TAC of one gram of *Sclerocarya birrea* fruit exocarp, expressed in mg of gallic acid equivalence per one gram of dry mass of an extract (mg GAE/g). Data

presented as mean $\pm$ standard error ($n = 3$). Significant differences ($p < 0.05$) are indicated by letters above the bars, where different letters depict significant differences between average means. The same letters indicate that means are not significantly different (**D**)

highest DPPH and FRAP reduction with $27.21 \pm 0.31\%$ and $29.53 \pm 0.12\%$ respectively. For ABTS assay, Hexane extracts recorded the 3rd highest inhibition of $15.43 \pm 0.94\%$ at 1 mg/mL. Hexane extracts recorded the least inhibition with $27.21 \pm 0.31\%$ and $8.67 \pm 0.01\%$ for DPPH and FRAP assays, while Chloroform extracts recorded the lowest ABTS reduction with $13.27 \pm 1.69\%$ at 1 mg/mL. Figure 2D shows that Methanol extracts showed the highest TAC with a value of 41.43 ± 1.65 mg GAE/g, followed by ethyl acetate extracts and chloroform extracts with 38.3 ± 1.13 and 19.86 ± 1.02 mg GAE/g respectively. Hexane extracts had the lowest TAC value of 7.1 ± 0.2 mg GAE/g. The TAC of Ethyl acetate and Methanol extracts were not significantly ($p < 0.05$) different from each other but were significantly different from hexane and chloroform.

At the lowest concentration (0.0312 mg/mL), Methanol extracts recorded the highest inhibition with $36.53 \pm 3.53\%$ for DPPH, $18.86 \pm 0.08\%$ for FRAP and $17.08 \pm 1.61\%$ for ABTS compared to recordings of gallic acid which were $39.26 \pm 0.92\%$, $15.41 \pm 0.10\%$ and 24.99 ± 1.17 for DPPH, FRAP and ABTS respectively. Ethyl acetate extracts showed the 2nd lowest antioxidant activity with $19.51 \pm 1.67\%$ (DPPH), $3.82 \pm 0.12\%$ (FRAP) and $15.23 \pm 1.56\%$ (ABTS) inhibitions. Chloroform extracts recorded the 3rd reduction with $4.65 \pm 0.36\%$ (DPPH), $2.76 \pm 0.19\%$ (FRAP), $7.75 \pm 1.26\%$ (ABTS). Hexane extracts recorded the lowest antioxidant activity at 0.0312 mg/mL with $1.61 \pm 0.21\%$, $0.57 \pm 0.01\%$ and $6.61 \pm 1.05\%$ for DPPH, FRAP and ABTS inhibitions respectively.

Cytoprotective effects of *Sclerocarya birrea* fruit exocarp extracts against H_2O_2 -induced cell death

In the positive control experiment, untreated Hela cells were exposed to 1 mM of H_2O_2 , all cell except $27.3 \pm 0.23\%$ were dead. At the lowest extract concentration (12.5 μ g/mL), Hexane extracts did not significantly protect cells from H_2O_2 -induced cell death with only $32 \pm 3.12\%$ cells remaining viable. Cell protection significantly increased with increasing solvent polarity, with the protection progressing from Chloroform to Ethyl acetate, and Methanol extracts recorded the highest protection with $74 \pm 2.61\%$ of HeLa cells. However, Methanol extracts as the best protective agents were significantly less effective than of negative control (cells treated with PBS only and not exposed to H_2O_2) which registered $98.08 \pm 0.6\%$ viable cells at the lowest concentration. The cell protection generally increased with polarity and concentration. At the highest extract concentration (400 μ g/mL), all extracts significantly ($p < 0.05$) protected cells compared to positive control. Ethyl acetate extracts had the highest protective effect by protecting $94 \pm 2.3\%$ cells which was as effective (no significantly different) as in the

negative control. This was followed by Methanol extracts with $92.2 \pm 0.2\%$ of cells, then Chloroform extracts. Hexane extracts showed the least protectiveness with $78 \pm 2.96\%$ viable cells at the highest concentration (Fig. 3).

Effects of extracts on the SOD, CAT and GSH activities

Figure 4, B show that Methanol extracts showed the highest effect amongst all extracts with 9.61 ± 0.44 U/mL and 49.18 ± 2.39 U/mL for SOD and CAT activities which is significantly higher than untreated cells (control) which recorded 4.68 ± 0.85 U/mL and 33.17 ± 0.82 U/mL for SOD and CAT respectively. Extracts showed proportionality between solvent polarity and SOD and CAT activities. Ethyl acetate extracts showed the 2nd highest effect with 9.22 ± 0.50 U/mL, 42.66 ± 3.30 U/mL for SOD and CAT respectively. However, there were no significant differences in the SOD and CAT activities from cells treated with Methanol and Ethyl acetate extracts. Chloroform extracts recorded the 3rd highest effect in both SOD and CAT activities with 7.68 ± 0.60 U/mL and 35.54 ± 42.33 U/mL respectively. It is important to note that activity of SOD treated with Chloroform extracts were significantly higher than that in control cells, while in the CAT activity experiment, it was numerically higher but not significantly higher. Hexane extracts recorded values higher than that of control but not statistically different for SOD, CAT and GSH activities with 5.41 ± 0.41 U/mL, 33.70 ± 0.82 U/mL and 21.03 ± 0.89 mmol/L mmol/L respectively. Control cells showed GSH activity of 18.97 ± 1.64 mmol/L, which was significantly lower than the activities in cells treated with Ethyl acetate extracts 35.19 ± 1.97 mmol/L > methanol extracts > chloroform extracts as shown in Fig. 4C

In silico studies

Molecular docking and types of interactions

To determine if compounds from *Sclerocarya birrea* can act as agonists of the SOD and CAT antioxidant enzymes, phytochemicals of *Sclerocarya birrea* were obtained from literature and subjected to molecular docking (Table 1). Table 2 shows that of the 10 compounds reported to be from *Sclerocarya birrea*, Myricetin 3-o- α - rhamnoside (C8) showed the lowest binding affinity (-9.1 kcal/mol) to the active site of SOD. Gallic acid (C1) on the other hand showed the highest binding affinity at -6.1 kcal/mol. As for CAT, Myricetin 3-o- α - rhamnoside (C8) showed a binding affinity of -8.8 kcal/mol which was the lowest amongst all the other compounds as shown in Table 1. Molecular interactions determined from 2-dimensional structures of

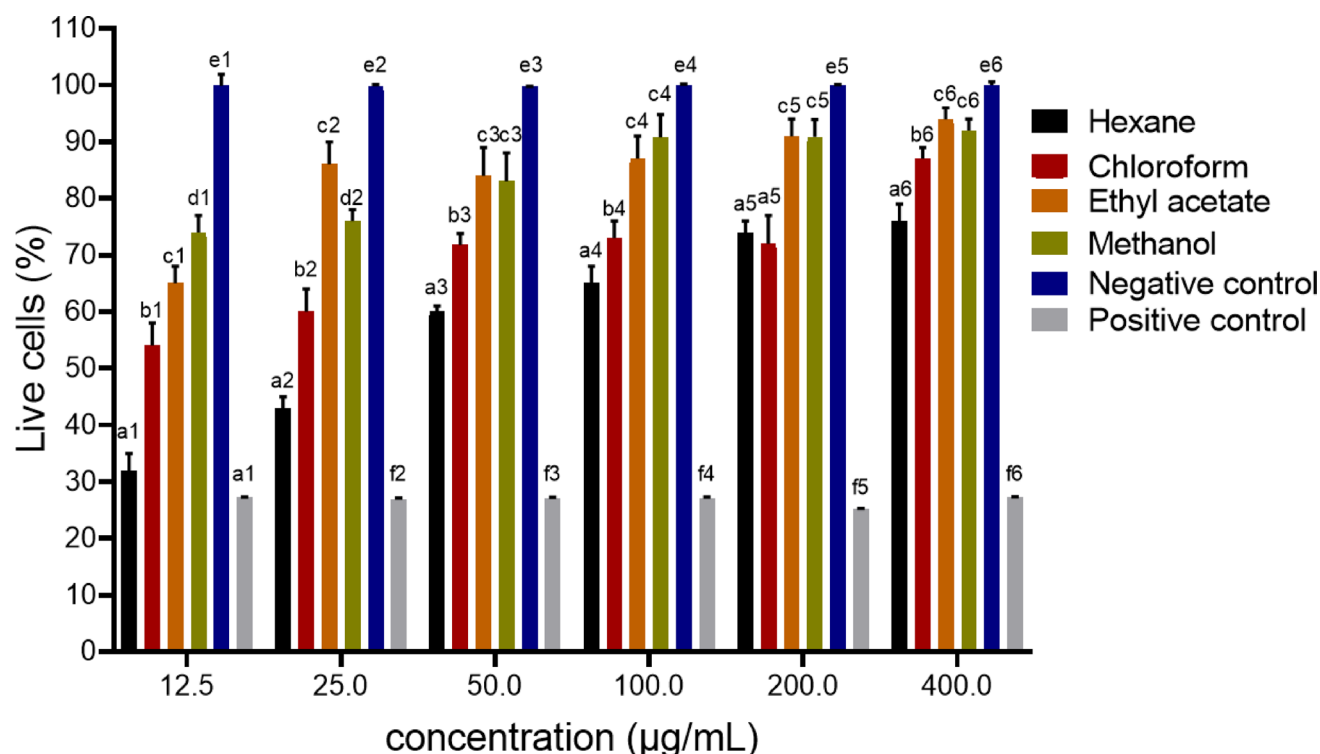


Fig. 3 Cytoprotective effect of *Sclerocarya birrea* fruit exocarp extracts against H_2O_2 -induced cell death. Negative control = untreated HeLa cells + PBS only. Positive control = HeLa cells exposed to 1 mM H_2O_2 only. Data is presented in mean $\pm$ standard error ($n = 3$). The

letters above bars indicate significant differences ($p < 0.05$) between means. The numbers attached to the letters indicate a comparison between means in the same concentration

Myricetin 3- α -rhamnoside (C8) in complex with SOD and CAT enzymes was elucidated using Biovia Discovery Studio software. Shown in Fig. 5a, Myricetin 3- α -rhamnoside (C8) attached to the active site of SOD enzyme and formed hydrogen bonds with 3 amino acids; HIS A:61, HIS A:118 and GLY A:139, van der Waals forces and π -alkyl/ π - π stacked electrostatic interactions with key amino acids of the SOD catalytic site as shown in Fig. 5b. However, Myricetin 3- α -rhamnoside (C8) also formed unfavourable bonds with HIS A: 46, ARG A: 141, THR A:135, and HIS A: 44 of SOD. Figure 6 shows the molecular docking interactions between Myricetin 3- α -rhamnoside and CAT. Where hydrogen bonding to 4 amino acids; ARG A:91, VAL A:125, ALA A:311, and PHEA: 313 were observed. Other interactions such as van der Waals, alkyl/ π -alkyl and π -cationic interactions were observed as shown in Fig. 6b.

Discussion

With many non-contagious diseases being linked to metabolic disorders, plants are now occupying the center of attention as a potential for solutions for oxidative stress-based diseases. Therapies therefore, require the use of substances with the capacity to reduce highly reactive radicals

such as those that arise from oxygen-based reactions. It has been shown that plant extracts, through electron and hydrogen transfer, reduce ROS. It has also been shown that some endogenous antioxidants are restored by plant extracts. To study the potential of *Sclerocarya birrea* fruits exocarp as an efficient source of antioxidants, we needed to start with quantitative phytochemical analysis of the total flavonoid content (TFC) and total phenolic content (TPC) of extracts. Flavonoids and phenols are known sources of antioxidants, and Methanol extracts showed the highest TFC and TPC with 55.25 ± 3.8 mg QE/g and 105 ± 1.4 mg GAE/g, respectively (Fig. 1A, B). These findings are supported by Manzo et al. (2017) and Russo et al. (2018), who reported substantial levels of flavonoids and phenolic contents in *Sclerocarya birrea*. Flavonoids and phenolics are groups of metabolites encompassing saponins, tannins, terpenoids, cardiac glucoside, and steroids that have been reported in the stem bark, roots, and leaves of *Sclerocarya birrea* (Virginie et al. 2016; Manzo et al. 2017; Russo et al. 2018). However, it is important to note that the proportionality and differences in TFC and TPC observed between extracts of different solvents may be attributed to by solvent polarity. Different solvents have different extraction strengths, and therefore, different efficiencies (Aruwa et al. 2019; Zullai-kah et al. 2019). The other contributing factor might be the

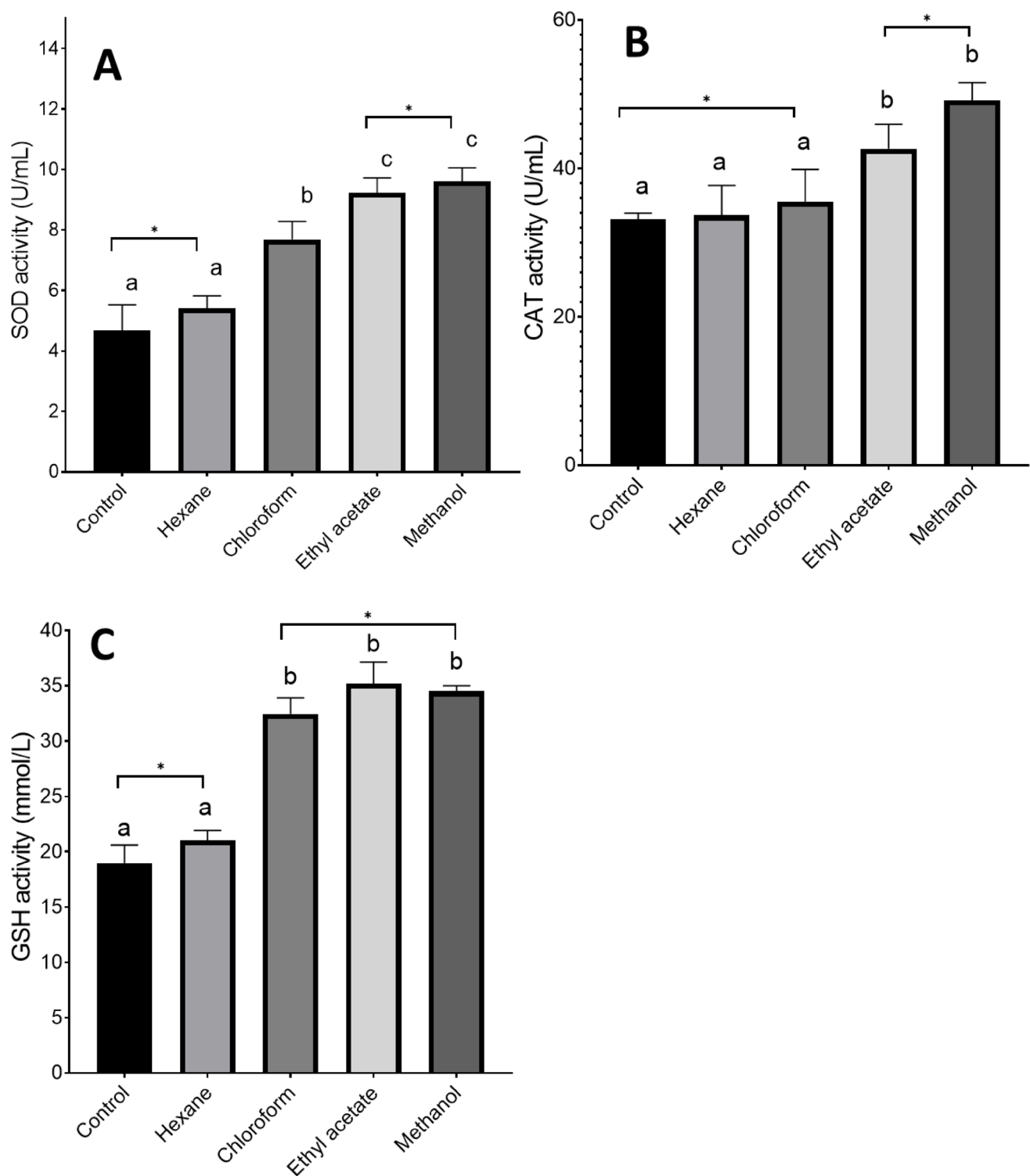


Fig. 4 Effects of extracts of *Sclerocarya birrea* fruit exocarp on **A** superoxide dismutase (SOD), **B** catalase (CAT), **C** glutathione (GSH) activities on 90% confluent HeLa cells at passage number 5. Control = SOD, CAT, GSH activities on untreated HeLa cells. Data presented

as mean $\pm$ standard error ($n = 3$). Significant differences ($p < 0.05$) are indicated by letters above the bars, where different letters depict significant differences between average means. The same letters indicate that means are not significantly different

Table 1 IC₅₀ values of *Sclerocarya birrea* fruit exocarp extracts

Antioxidants assay	IC ₅₀ (mg/mL)			
	Hexane	Chloroform	Ethyl acetate	Methanol
DPPH	>1	>1	0.89	0.12
FRAP	>1	>1	0.43	0.09
ABTS	>1	>1	0.29	0.25

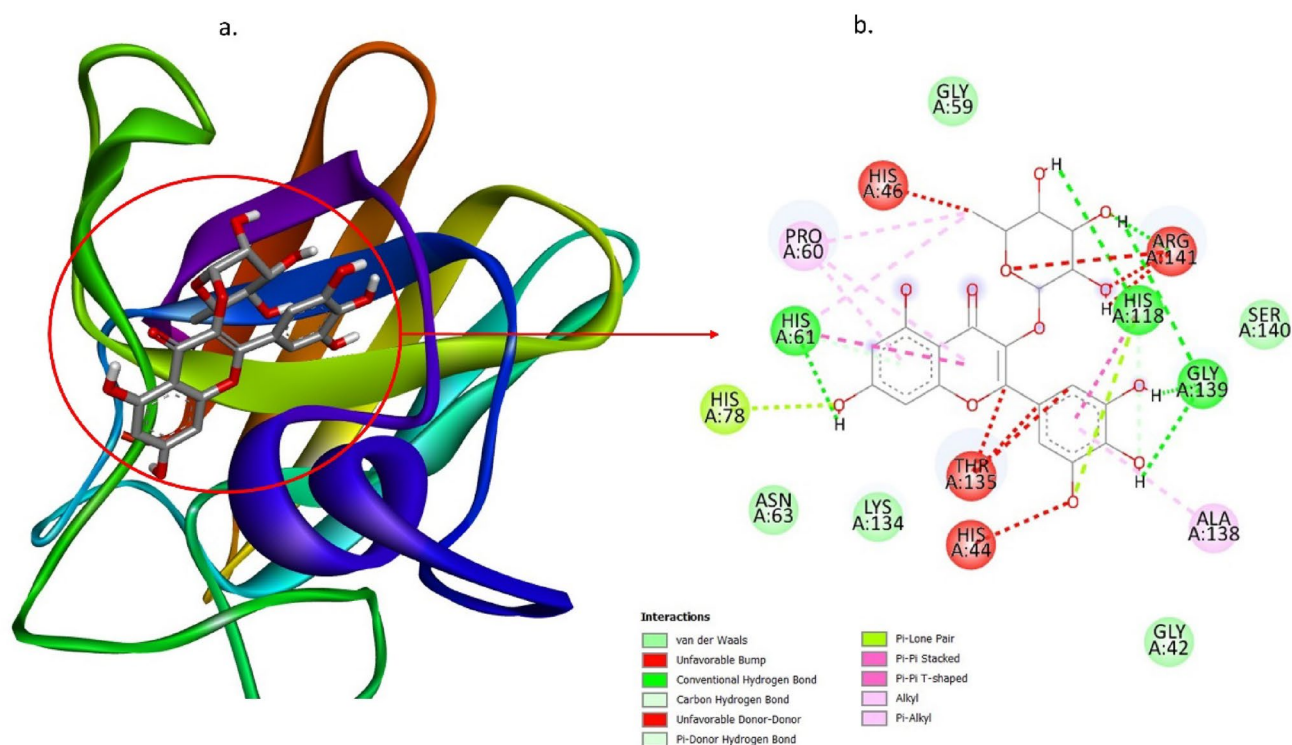
Table 2 Binding affinity scores for *Sclerocarya birrea* compounds on Superoxide dismutase (SOD) and Catalase (CAT)

Compound ID	Compound name	PubChem ID	Binding Affinity (Kcal/mol)	
			SOD	CAT
C1	Gallic acid	370	-6.1	-6.0
C2	Epicatechin gallate	107905	-8.9	-8.5
C3	Quercetin	5280343	-8.1	-8.0
C4	Quercetin 3-o-glucopyraside	5280804	-8.6	-8.5
C5	Quercetin 3-o- α -L-rhamnoside	5280459	-8.9	-8.6
C6	Quercetin 3-o- α -D-arabinofumanoside	11968848	-8.6	-7.7
C7	Quercetin 3-o- α -D-galactopyranoside	5281643	-9.0	-7.9
C8	Myricetin 3-o- α -rhamnoside	5281673	-9.1	-8.8
C9	Astragalin	5282102	-8.4	-7.9
C10	Epigallocatechin 3-o-gallate	65064	-8.9	-8.5

presence of higher levels and solubility of high polar phytochemicals (Xu et al. 2016).

ROS are neutralized by antioxidants, which are normally produced by the body (Gulcin 2020). Oxidative stress is a condition where antioxidants are at a concentration that does not effectively neutralise ROS (Sies 2020). We then assessed radical scavenging activity in vitro. Methanol extracts showed the most reducing capacity when tested against DPPH, FRAP, and ABTS oxidants. DPPH and ABTS assays measure both Hydrogen Atom Transfer (HAT) and Single Electron transfer (SET), while FRAP and TAC measure purely SET. Methanol extracts recorded IC₅₀ values of 0.12 mg/mL, 0.09 mg/mL, and 0.25 mg/mL for DPPH, FRAP, and ABTS respectively (Fig. 2A–C). These observations are following those of Da Costa Mousinho et al. (2013) and Niang et al. (2021) findings. Figure 2D also showed that methanol extracts had the highest TAC with 41.43 ± 1.65 mg GAE/g, which is supported by Akoto et al. (2020). Higher antioxidants activity of methanol extracts are most probably due to higher flavonoid and phenolic contents compared to other extracts, as shown earlier.

Having evaluated the quantitative phytochemical analysis and radical scavenging activity, we investigated extracts for their capacity to prevent cell death induced by H₂O₂. Hydrogen peroxide oxidizes and disrupts cell membrane, as well as oxidize proteins, resulting in cell death (Armogida et al. 2012). When cells treated with extracts were subjected to H₂O₂, ethyl acetate had the highest protective effect at 400 μ g/mL, by protecting $94 \pm 2.3\%$ of cells. The ability of

**Fig. 5** A SOD docked with Myricetin 3-o- α -rhamnoside (C8). B types of interactions with residues

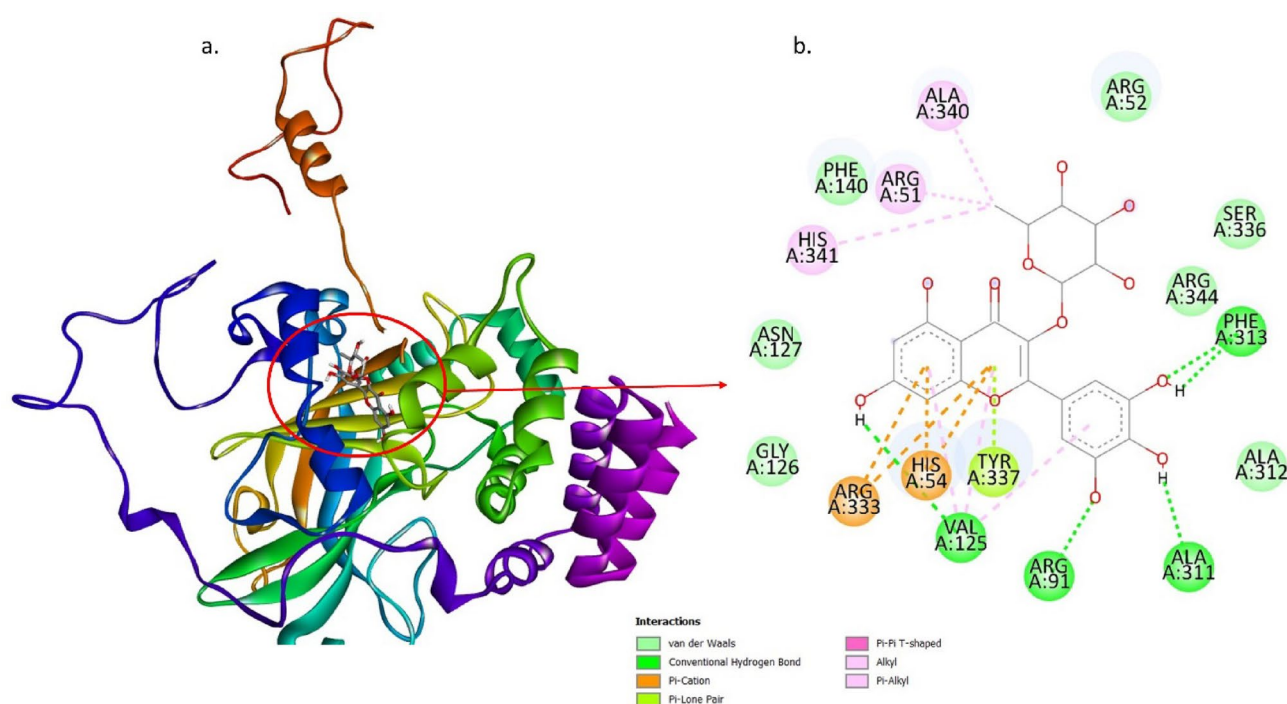


Fig. 6 **A** Catalase docked with Myricetin 3-o- α -rhamnoside (C8). **B** types of interaction with residues

extracts to significantly protect HeLa cells suggests that they can be developed into cellular oxidative stress protective and quenching agents with clinical implications. Effective cytoprotective effects by *Sclerocarya birrea* extracts have also been observed in other members of the family ANACARDIACEAE as reported previously (Noguera-Artiaga et al. 2019; Xanthis et al. 2021), where *Hydrosos pistachio* and *Pistacia lentiscus* effectively protected retinal pigment epithelial (RPE) cells, and human keratinocyte cells (HaCaT) cells from H_2O_2 induced cell death. The extracts may have transferred either hydrogen and/ or electrons to neutralize H_2O_2 , preventing damage to the membrane of HeLa cells thus protecting against oxidative stress mediated cell death. It also possible that the extracts perpetuated intercellular antioxidants activity by elevating SOD, CAT, and GSH activities. The SOD activity increased by 4.93 U/mL in cells treated with methanol extracts (Fig. 3A), CAT activity increased by 16.01 U/mL in cells treated with methanol extracts (Fig. 3B), and GSH activity increased by 16.22 mmol/L in cells treated by ethyl acetate extracts (Fig. 3C). The increase in SOD, CAT, and GSH activities after treatment by plant extracts, have been reported before (Ngueguim et al. 2015; Abubakar et al. 2021), where stem bark extracts of *Sclerocarya birrea* elevated activities of SOD, CAT, and GSH. Figures 5 and 6 show the predicted docking and residue interaction between Myricetin 3-o- α -rhamnoside (C8) and SOD and CAT. Myricetin 3-o- α -rhamnoside (C8) is a phenolic compound with flavonoid

moiety found in *Sclerocarya birrea* (Russo et al. 2013). C8 may have upregulated SOD activity by 2 Pi- Alkyl electrostatic interactions, 3 hydrogen bonds, and 1 Pi-Lone pair interaction with 6 residues of SOD. The interactions shown by the bonding of the compounds to SOD and CAT may suggest that phytochemicals from *Sclerocarya birrea* may act as agonists of the antioxidant enzymes and modulate their activities. The use of phytochemicals as agonists of SOD and CAT has been previously reported by Rana et al. (2019) and this supports the findings of this study.

Conclusion

Our results showed the order of total flavonoid and total phenolic contents were methanol>ethyl acetate>chloroform>hexane of *Sclerocarya birrea* fruits exocarp extract. ABTS, DPPH, FRAP and TAC activities showed antioxidant activity. All solvent extracts displayed effective protection in HeLa cells against H_2O_2 -induced cell death. Thus, flavonoid and phenolic extracts from *Sclerocarya birrea* fruits exocarp may function as free radical cleaners to reduce cellular ROS levels and prevent the formation of apoptosis cells caused by H_2O_2 oxidation. On the basis of the current studies, we conclude that flavonoids and phenolics of *Sclerocarya birrea* fruits exocarp improved antioxidant activity and thus have the potential to mitigate oxidative stress. We also showed upregulation of antioxidant enzymes

including SOD, CAT and non-enzymatic GSH activities with methanol and ethyl acetate extracts. These findings indicate that extracts of *Sclerocarya birrea* fruits exocarp might be useful functional ingredients in curbing oxidative stress, hence preventing human pathogenesis. We recommend further studies in isolating compounds and verifying their affinity to SOD, CAT and GSH antioxidants in vitro.

Acknowledgements We would like to thank Botswana International University of Science and Technology department of Biological Sciences and Biotechnology for the facilities.

Author's contributions Phazha Bushe Baeti, MSc—Conceptualizing, methodology, drafting, investigations, analyzing, software, reviewing & editing, validation, project administration, fund acquisition and writing the final paper. Phenyio Donald Brown, MSc and Keagile Bati, MSc—Investigation, writing, methodology, analyzing, reviewing & editing. Runner Majinda, PhD, Kabo Masis, PhD, Goabaone Gaobotse, PhD, and Tebogo Elvis Kwape, PhD—Conceptualizing, reviewing and editing, validation, Project administration, fund acquisition, and supervising.

Funding Open access funding provided by University of Botswana. This research was funded using Research grant: S00376 (Project grant code) issued by research office of Botswana International University of Science and Technology.

Declarations

Conflict of interest The authors have no competing interest do declare that are relevant to the content of this article.

Ethical approval Permission was granted by the Botswana government, Ministry of Health and Wellness permit code: HPDME 13/18/1.

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