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Phytochemical composition and chemical profiling of extracts of *Cordia grandicalyx* Oberm

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Background: The search for bioactive organic products for the treatment of various diseases is a growing concern worldwide, because these bioactive natural products are associated with fewer side effects and are easily accessible.

Aim: The present study analysed the phytochemical constituents and cytotoxic effects of the leaf, bark and fruit extracts of *Cordia grandicalyx* Oberm.

Setting: Plant samples were collected from GaMashishimale village in Ba-Phalaborwa Municipality, South Africa.

Methods: Antioxidant activity was determined through 1,1-diphenyl-2-picrylhydrazyl radical scavenging method, whilst cytotoxic assay was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide method. Plant extracts were subjected to phytochemical profiling using both qualitative and quantitative methods. The extracts were also subjected to fractionation using column chromatography and thin-layer chromatography, and the sub-fractions with considerable yields were identified using nuclear magnetic resonance (NMR).

Results: The aqueous extracts of the bark and leaves had significant ($p < 0.001$) antioxidant activity as compared to negative controls and ascorbic acid. Additionally, appreciable amounts of total phenolic and alkaloid contents were recorded on polar extracts, notably 200 mg/GAE and 140 mg/quercetin equivalents for leaf cold water and leaf hot water extracts, respectively. All extracts were not toxic to cells, whilst the positive control (H_2O_2) led to almost 100% demise of cells. Two compounds were isolated from the leaf acetone extracts collected from fraction 20 to 30 and fraction 101 to 120 and identified as α-amyrin and β-amyrin by NMR spectral analysis.

Conclusion: The study provided evidence supporting the screening of plants for the discovery of therapeutic compounds. The study also revealed that all the different *C. grandicalyx* extracts were less toxic to cells and may provide scientific backing for continued use of the plant in mixtures for the treatment of diseases.

Keywords: *Cordia grandicalyx* Oberm; amyrin; cytotoxicity; antioxidant activity; phytochemical compounds.

Introduction

The South African Medical Research Council (SAMRC) declared in a press release that about 60% of adverse drug effects in patients on prescription drugs admitted in hospitals are caused by traditional medicines (SAMRC 2018/2019). The report further asserts that co-usage of traditional medicines and prescription drugs for the treatment of diseases is common and might lead to health complications with heavy economic implications on the users (Ozioma & Okaka 2019).

Literature states that, prior to the 20th century, approximately 80% of all medicinal therapy used to treat human and animal illnesses was derived from medicinal plants (Ogbole, Segun & Adeniji 2017). Traditional knowledge of medicinal plants has provided useful leads for new compounds derived from medicinal plants (Gurib-Fakim 2006). Literature also reports that medicinal plants possess pharmacological properties such as antidiabetic, antimicrobial and antihyperlipidemic properties (Modak et al. 2007). Medicinal plants play an important role in the basic health needs of developing countries. South Africa has a vast range of important medicinal plants, and their therapeutic potency has been demonstrated by several researchers (Chauke et al. 2015; Steenkamp & Gouws 2006). Rural populations still rely on traditional systems for the treatment of different ailments, but safety and efficacy for some plant species are yet to be verified. Furthermore, their use by traditional healers does not hold a primary therapeutic function as the methods of preparation and dosing are different for each healer (Oziama & Okaka 2019; Ngobe, Semenya &

Sodi 2021). Many of the species reportedly used for the preparation of medicine and treatment of various diseases have no recorded evidence or data on clinical studies or cytotoxic activity (Alamgeer et al. 2018). Despite the lack of evidence to support the use of certain plants as medicine, about half of the drugs approved for disease treatment in many countries are either plant-derived natural products or their derivatives (Dias, Hamerski & Pinto 2010). This therefore justifies studies on medicinal plants to overcome the growing problem relating to ineffective medicines for the treatment of diseases.

Research on medicinal plants is ongoing, and this information has led to some promising evidence on potential of medicinal plants as present and future remedies. (Akhalwaya, Van Vuuren & Patel 2018; Tandon & Yadav 2017). A few therapeutic effects such as anti-inflammatory, antimicrobial and antioxidant properties have been reported under the genus *Cordia* (Gurib-Fakim et al. 2000; Oza & Kulkarni 2017), but nothing has been reported on *Cordia grandicalyx* Oberm. *Cordia grandicalyx* Oberm is used, albeit rarely, as an antidiabetic medicinal plant by traditional healers of Ga-Mashishimale village in Phalaborwa, South Africa (Chauke et al. 2015). It belongs to the family Boraginaceae and is predominantly found in the eastern parts of South Africa. The Boraginaceae family comprises about 135 genera with about 2600 species distributed throughout the tropical, subtropical and temperate regions of the world (Oza & Kulkarni 2017).

Chauke et al. (2015) demonstrated that the plant species had promising antioxidant and glucose uptake-enhancing activities. Furthermore, the genus *Cordia* has been reported to have several therapeutic effects such as antioxidant, antidiabetic and anti-inflammatory (Oza & Kulkarni 2017). This study was aimed at investigating the cytotoxic effects of the fruit, bark and leaf extracts of *C. grandicalyx* on muscle, preadipocyte, Vero and liver cells, as well as to establish the phytochemical composition of the different crude extracts.

Research methods and design

Study setting and preparation of plant material

Fruits, leaves, bark and roots of *C. grandicalyx* were harvested from the Ba-Phalaborwa municipality in a village called Ga-Mashishimale, South Africa, and were coded A1, A2, and A3 respectively. The prototype was deposited in the H.G.W.J. Schweickerdt Herbarium at the University of Pretoria.

Sampling and preparation of extracts for screening

The plant samples were pulverised using a Polymix laboratory mill (PX-MFC 90D, Kinematica) into a fine powder. The powders were stored in clean sterile bottles at room temperature until the extraction procedure. Powders (5 g) of the different plant materials were extracted with 20 mL of different solvents of varying polarities, viz., *n*-hexane (SMM Instruments), chloroform (Merck), ethyl acetate (SMM Instruments) and acetone (SMM Instruments). To maximise the extraction of phytochemicals, plant materials were soaked in the appropriate solvent for at least 24 h at room temperature. The extracts were purified using Whatman No. 1 filter paper, and the filtrates were transferred into preweighed glass beakers. The filtered extracts were left to dry in a stream of cold air at room temperature to ensure that volatiles were retained in the extracts. A working concentration of 10 mg/mL was prepared from the dried filtrates in 10% (*v/v*) dimethyl sulfoxide (DMSO) (Merck SA) and phosphate-buffered saline (PBS), pH 7.4.

The plant materials were also extracted with water. Approximately 50 g of the different powdered plant materials were extracted in 1000 mL distilled water (cold water, hot water, boiled for 30 min). The samples were filtered and frozen at −20 °C overnight and then freeze-dried *in vacuo* using a freeze dryer (VirTis freeze-dryer, SP Scientific) for 4 days. The percentage yield of each freeze-dried extract was weighed up and recorded. A working solution of 10 mg/mL was prepared by dissolving the extracts in 10% DMSO in PBS. The extracts used in the study were BB (bark boiled), BHW (bark hot water), BCW (bark cold water), BA (bark acetone), BEA (bark ethyl acetate), BC (bark chloroform), BH (bark hexane), FB (fruits boiled), FA (fruits acetone), FEA (fruits ethyl acetate), FC (fruits chloroform), FH (fruits hexane), LB (leaf boiled), LHW (leaf hot water), LCW (leaf cold water), LA (leaf acetone), LEA (leaf ethyl acetate), LC (leaf chloroform), LH (leaf hexane) and Leave extracts compound (LCMP) (β-amyrin). Controls are represented by NEG (negative control) and POS (insulin, metformin and ascorbic acid).

Data collection

The experiments were performed in triplicate using three biological replicates ($n = 3$) in three independent experiments, and data was presented as mean ± standard deviation.

Data analysis

The experimental samples were compared with the negative and positive controls. A two-way ANOVA was used to determine student *t*-test and confidence intervals. The differences between groups were considered significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Qualitative phytochemical analysis

All phytochemical tests were performed according to the standard methods as described by Parekh and Chanda (2007) and Harbone (1998). Qualitative phytochemical screening of the different extracts was performed for the detection of alkaloids, flavonoids, terpenoids, cardiac glycosides, proteins, steroids, terpenes, tannins and terpenoids.

Alkaloids

The dried extract (1 g) was dissolved in 5 mL of 2N HCl and purified using a Whatman No. 1 filter paper. The filtrate was treated with an equal amount of Dragendorff's reagent

(Rochelle Chemicals). The presence of an orange precipitate indicated the presence of alkaloids.

Steroids

Each extract was added to 10 mL of chloroform and an equal volume of concentrated sulphuric acid was added by the sides of the test tube. The presence of steroids was confirmed in the sample when the upper layer turned red, with the sulphuric acid layer turning yellow with green fluorescence.

Terpenoids

Five millilitre of specific extract was mixed with 2 mL of chloroform and 3 mL of concentrated sulphuric acid. A deep reddish-brown colour indicated the presence of terpenoids.

Tannins

The extract (1 mL) was mixed with 1 mL of a 1% alcoholic $FeCl_3$. A brownish-green or a blue-black precipitate indicated the presence of tannins.

Cardiac glycosides

Each extract was mixed with 2 mL of glacial acetic acid containing one drop of 5% ferric chloride solution. This mixture was under-layered with 1 mL of concentrated sulphuric acid. The appearance of a greenish-blue colour within a few minutes indicated the presence of cardiac glycosides.

Flavonoids

Five drops of 1% aluminium chloride solution were added to a 1 mL of extract. A yellow colouration indicates the presence of flavonoids.

Proteins

One millilitre of each extract was mixed with 1 mL concentrated sulphuric acid. The presence of a white precipitate indicated the presence of proteins.

Sterols and terpenes

The extracts were shaken with 1 mL petroleum ether to remove the colouring material. The residues were reconstituted with 10 mL chloroform, and the chloroform layer dried over anhydrous sodium sulphate. Chloroform (5 mL) layer was mixed with 0.25 mL of acetic anhydride and then two drops of concentrated sulphuric acid were added. The green colour indicated the presence of sterols, whilst pink to purple indicated the presence of terpenes.

Quantitative phytochemical determination

Quantitative phytochemical determination of the extracts was performed.

Estimation of total phenolic contents

Estimation of total phenolic contents was performed according to the method of Singleton, Orthofer and Lamuela-Raventós (1999), with slight modifications. Approximately 25 µL of the different plant extracts (10 mg/mL) was oxidised using Folin-Ciocalteau's phenol reagent (250 µL), and the reaction incubated at room temperature for 5 min. Briefly, 750 µL of 20% anhydrous sodium carbonate was added to the mixture to stop the reaction. Distilled water was added to the reaction mixture to a total volume of 5 mL and further incubated for 15 min at room temperature. Estimation of total phenolic contents was measured using a spectrophotometer at 760 nm, and results were deduced from a gallic acid calibration curve (0–500 µg Gallic acid). Phenolic content was expressed as mg gallic acid equivalent (GAE)/g extract, which was calculated as follows:

$$C = \frac{c.V}{m} \quad \text{[Eqn 1]}$$

C is the total content of phenolic compounds in mg GAE/g plant extract; c is the concentration of gallic acid extrapolated from the calibration curve, mg/mL; V is the volume of extract in mL and m the weight of plant extract in g.

Estimation of total flavonoids contents

The aluminium colorimetric method was used for determination of the total flavonoids as described by Chang et al. (2002). Approximately 25 µL of the plant extract (10 mg/mL) was mixed with 750 µL of methanol, 50 µL of 10% aluminium chloride (Saarchem Chemicals), 50 µL of a 1 mol/L sodium acetate and 1.4 mL of deionised water. The redox reaction was left in the dark at room temperature for 30 min. The absorbance of the redox reaction was read at 420 nm. Total flavonoids contents were determined from a quercetin standard curve and expressed as mg quercetin equivalents/g, which was calculated as concentration using the following formula:

$$C = \frac{c.V}{m} \quad \text{[Eqn 2]}$$

Estimation of total tannin contents

Tannin content was analysed according to the method as described by Marinova, Ribarova and Atanassova (2005). Approximately 0.1 mL of the extract was mixed with 7.5 mL deionised water and 0.5 mL Folin-Ciocalteu phenol reagent (Sigma Aldrich SA), 1 mL 35% sodium carbonate solution, and diluted to 10 mL with deionised water. The concoction was kept at room temperature, swirled for 30 min and the absorbance was read at 725 nm. The tannin content was expressed as mg GAE/g of extract, which was calculated as follows:

$$C = \frac{c.V}{m} \quad \text{[Eqn 3]}$$

Antioxidant activity

Qualitative analysis

Qualitative antioxidant activity was performed according to the method of Masoko et al. (2019). The plant extracts were

loaded onto the thin-layer chromatography (TLC) plates at 10 µg each and developed in TEA (Toluene: ethyl acetate: acetone, [9:1:0.1]). The plates were dried and then sprayed with 0.025% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol solution and allowed to develop for 5 min. The plates were observed for the emergence of yellow spots indicative of antioxidant activity.

Quantitative analysis

The quantitative antioxidant activity of plant extracts was determined through free radical scavenging activity using DPPH according to the method of Chauke et al. (2012). Plant extracts (10 mg/mL) were serially diluted in 96-well microtitre plates using aseptic deionised water (100 µL total volume). Then 100 µL 0.025% DPPH in methanol was added, followed by incubation at room temperature for 30 min. The reduction of absorbance of DPPH was determined at 540 nm. In negative control wells, 100 µL distilled water replaced plant extracts and 100 µL ascorbic acid (10 mg/mL) was used as a positive control. Colour controls, which contained dilutions of plant extracts, with 100 µL distilled water replacing DPPH, were used to account for the background absorbance of extracts. 2,2-Diphenyl-1-picrylhydrazyl radical has a deep violet colour in the solution, and it becomes colourless or pale yellow in the presence of a reducing agent. This property allows quantitative monitoring of the reaction at 520 nm. The corrected absorbance was calculated by subtracting background absorbance from corresponding test readings. Antioxidant activity was calculated as follows:

$$\% \text{ Antioxidant activity} = \frac{A_{control} - A_{test}}{A_{control}} \times 100 \quad \text{[Eqn 4]}$$

where A_{test} is the absorbance of the test sample and $A_{control}$ is the absorbance of the control sample. The IC_{50} values (mg/mL) were estimated from concentration vs. antioxidant activity graphs (Chauke et al. 2012).

Fractionation and isolation of compounds

Fractionation of the leaf extracts was performed according to the method by Kundu et al. (2016) with slight modifications. Two hundred grams of *C. grandicalyx* leaves were extracted in 1 L acetone, purified through a Whatman No. 1 filter paper. The filtrate was made into a homogenous paste by the addition of 20 g silica gel and evaporated on the rotary evaporator. The dried mixture was loaded onto a column packed with silica gel and eluted with *n*-hexane, followed by an increasing gradient of dichloromethane (DCM) (10% – 100%) in hexane, increasing gradient of ethyl acetate (10% – 100%) in DCM and methanol (10% – 20%) in ethyl acetate (500 mL each solvent mixture). The eluents (5 mL) were collected into test tubes and concentrated by evaporation at room temperature. The collected samples (240 test tubes) were separated on the TLC plates using TEA. The chromatograms were then visualised using vanillin/H_2SO_4 spray reagent and photographed under UV light at 254 nm. The tubes with similar TLC profiles were pooled together and subsequently analysed using nuclear magnetic resonance (NMR) spectroscopy.

Nuclear magnetic resonance

Nuclear magnetic resonance was performed according to the method of Abubakar and Haque (2020). Isolated compounds were dissolved in CD_3OD and the ^1H and ^{13}C NMR spectra were recorded using a Varian Gemini-300 NMR spectrometer. The system captured the two-dimensional records (2D) NMR for HMBC, HMQC, COSY proton and NOSY.

Cytotoxicity

Cytotoxic activity was measured according to the method of Ogbole et al. (2018) with slight modifications. Murine C2C12 myoblasts, 3T3-L1 preadipocytes, Vero monkey kidney and liver (H4IIE) cells were used. Cells were cultured in DMEM supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin cocktail and incubated at 37 ºC in a 5% CO_2 humidified incubator. The cells were passaged twice or thrice weekly to maintain the density of between 1×10^5 and 1×10^6 cells/mL. Cells (200 µL) were treated in 96-well plates with varying concentrations of *C. grandicalyx* extracts and isolated compound, with 5% (*w/v*) H_2O_2 used as a positive control, and incubated for 24 h. Then 20 µL 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (5 mg/mL in PBS) was added to cells in wells and incubated for a further 4 h at 37 °C. The medium was discarded and the formazan solubilised using 0.1 mL 0.04 N HCL in isopropanol, resulting in a purple-coloured solution. The absorbance of this coloured solution was measured at 570 nm using a Multiskan GO (ThermoScientific).

Ethical considerations

This article followed all ethical standards for research without direct contact with human or animal subjects.

Results

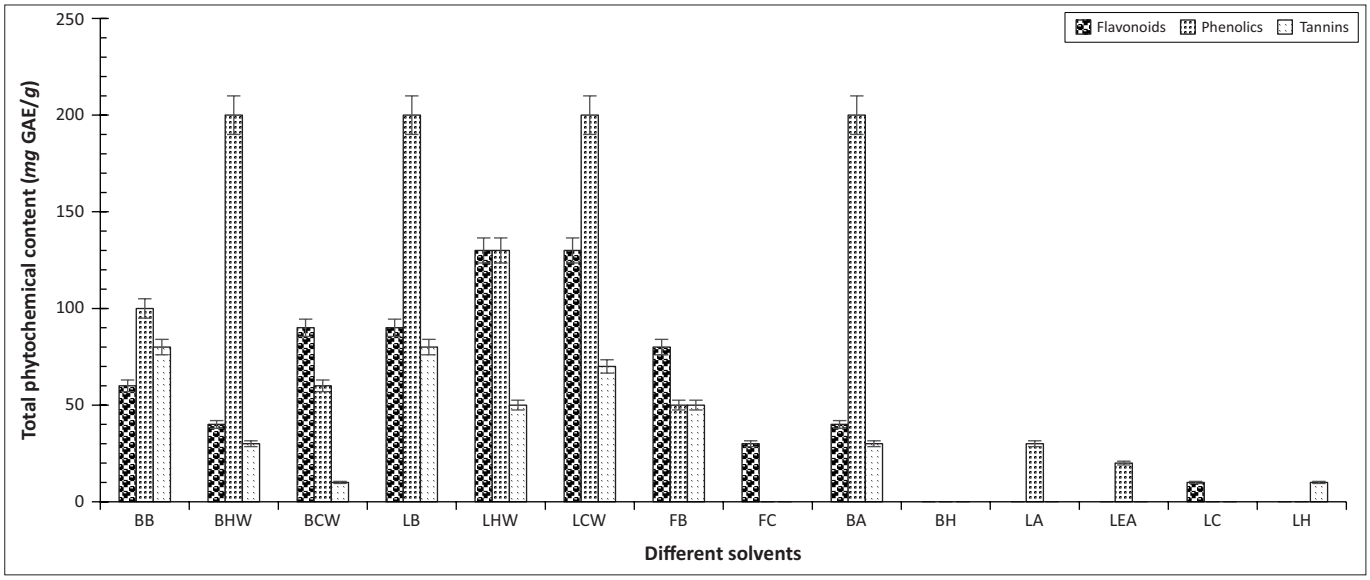
Phytochemicals analysis

Phytochemical analysis was performed through qualitative and quantitative screening methods. Preliminary qualitative phytochemical examination results are presented in Table 1. Alkaloids, cardiac glycosides, tannins and terpenoids were the most prominent in the different extracts. Alkaloids were present in all extracts but BH, LEA, LC and LH extracts. Anthraquinones and steroids were undetectable in all extracts, whilst phlobatannins were only detected in LEA. A minimal amount of flavonoids was observed, mainly with LA, LC and LH and BH. The different extracts had slight differences in the phytochemical constituents, and it was evident that both the bark and leaves contained relatively higher amounts of phytochemicals. Similarly, polar extracts had the most phytochemicals as compared to those extracted by other solvents.

TABLE 1: Qualitative phytochemical screening of the different *C. grandicalyx* extract, results reported as standard deviation ± mean of three replicates.

Phytochemicals	BB	BHW	BCW	LB	LHW	LCW	FB	BA	BH	LA	LEA	LC	LH
Alkaloids	+	+	+	+	+	+	+	+	–	+	–	–	–
Anthraquinone	–	–	–	–	–	–	–	–	–	–	–	–	–
Cardiac glycosides	+	+	+	+	+	+	+	+	–	–	–	–	–
Flavonoids	–	–	–	–	–	–	–	–	+	+	–	+	+
Phlobatannin	–	–	–	–	–	–	–	–	–	–	+	–	–
Steroids	–	–	–	–	–	–	–	–	–	–	–	–	–
Tannins	+	+	+	+	+	+	+	+	–	–	–	+	–
Terpenoids	+	–	+	+	+	+	+	–	–	–	–	–	–

BA, bark acetone; BB, bark boiled; BHW, bark hot water; BCW, bark cold water, BH, bark hexane; FB, fruits boiled; LB, leaf boiled; LHW, leaf hot water; LCW, leaf cold water; LA, leaf acetone; LEA, leaf ethyl acetate; LC, leaf chloroform; LH, leaf hexane.



BB, bark boiled; BHW, bark hot water; BCW, bark cold water; LB, leaf boiled; LHW, leaf hot water; LCW, leaf cold water; FB, fruits boiled; FC, fruits chloroform; BA, bark acetone; BH, bark hexane; LA, leaf acetone; LEA, leaf ethyl acetate; LC, leaf chloroform; LH, leaf hexane.

FIGURE 1: Total flavonoids (mg quercitin E/g), total phenolic and total tannin (mg GAE/g) contents of leaf, bark and fruit extracts of *C. grandicalyx.*

Total phenolic, tannin and flavonoids in these extracts were quantified. The phytochemical constituents displayed more phenolics, tannins and flavonoids compared to extracts of hexane and other non-polar solvents. The results revealed an increased amount of flavonoids for BB, BHW, BCW, LB, LHW, LCW, FB and BA extracts (Figure 1), with LCW and LHW recording 130 and 140 mg QE/g of dried extract, respectively.

Similarly, phenolic contents were pronounced in BHW, LB, LCW and BA, estimated at 200 mg GAE/g for all. Total condensed tannins were detected in BB, BHW, BCW, LB, LHW, LCW, FB and BA extracts (Figure 1), with BB and LB recording the highest tannin content at 80 mg GAE/g. Comparatively, the polar extracts contained notable phytochemicals, with leaves yielding higher constituents compared to the bark and fruits.

Antioxidant activity

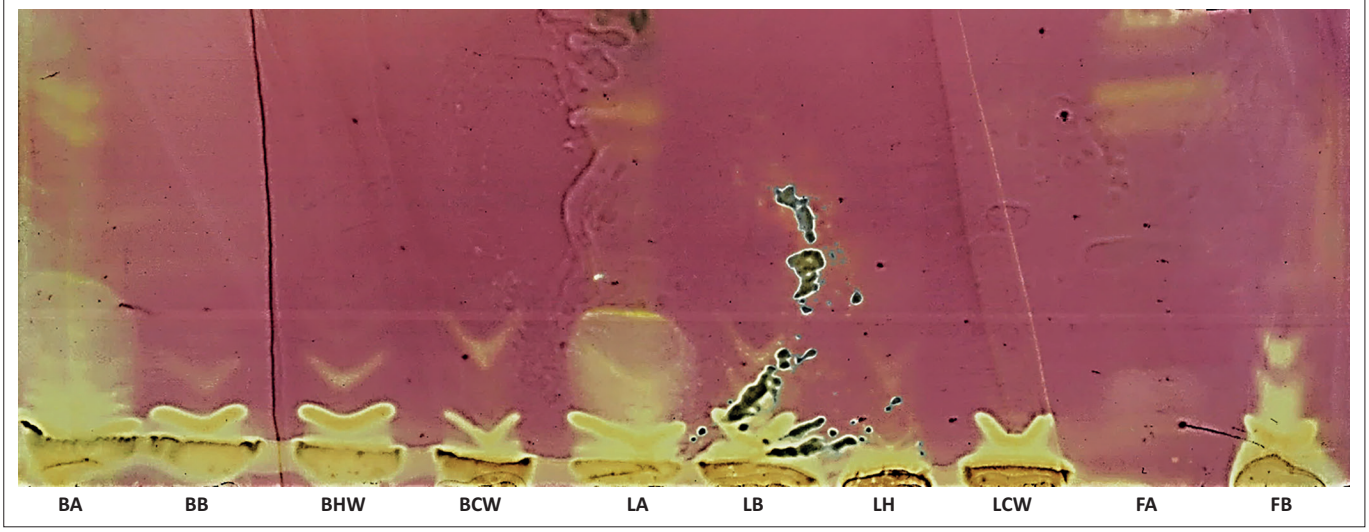
Qualitative antioxidant analysis using DPPH revealed promising antioxidant activity, as evidenced by clearing on a purple background on the TLC plate (Figure 2). However, the number of constituents exhibiting antioxidant activity varied amongst the different solvents. Amongst the extractants used in this study, polar extracts were the most effective.

Quantitative antioxidant activity was evaluated from the IC_{50} value, where antioxidant agents with high scavenging activity have $IC_{50} < 5$ mg/mL (Abdillah et al. 2015; Zhang, et al. 2015). Bark chloroform and leaves chloroform (LC) extracts exhibited comparable antioxidant activity to that of ascorbic acid at varying concentrations. The extracts demonstrated a dose-dependent decrease in oxidised DPPH concentration, with the highest concentration (2.5 mg/mL) exerting the most activity.

Most extracts recorded significant antioxidant activity ($p < 0.001$), mostly with the lowest concentration, as compared to that of the negative control (Figure 3). Antioxidants provide resistance against oxidative stress through their scavenging activity, and the antioxidant activity of medicinal plants are often directly linked to phenolics, tannins and flavonoids contents (Marreiro et al. 2017; Sembiring et al. 2018).

Compounds

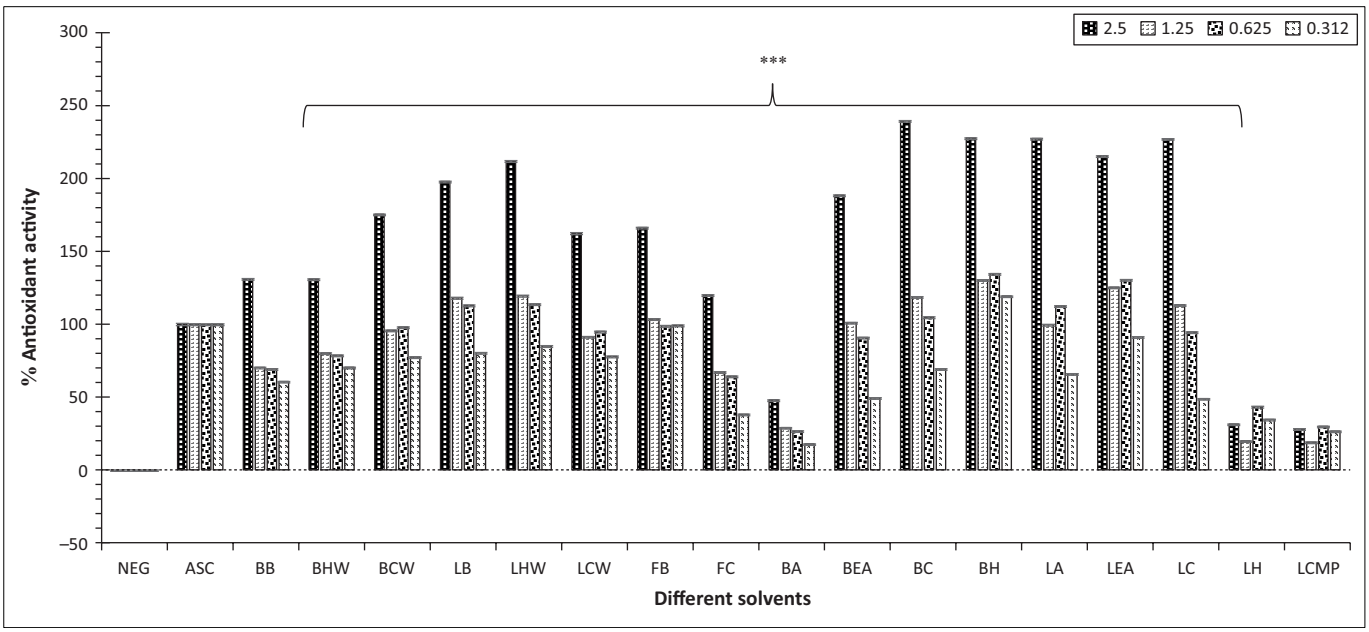
The silica-packed column was eluted at a flow rate of 5 mL/min as described in the methodology and 240 tubes were collected. The different fractions were analysed on TLC plates and similar fractions combined. Two pure compounds were isolated, viz. Compound 1 (0.5 g) collected from tubes 31 to 40 and Compound 2 (0.05 g) collected from tubes 101 to 120.



BA, bark acetone; BB, bark boiled; BHW, bark hot water; BCW, bark cold water; LA, leaf acetone; LB, leaf boiled; LH, leaf hexane; LCW, leaf cold water; FA, fruits acetone; FB, fruits boiled. DPPH, 2,2-diphenyl-1-picrylhydrazyl.

Note: The yellow colour on the purple background indicates the presence of antioxidant activity.

FIGURE 2: Antioxidant (radical scavenging) activity using diphenyl-1-picrylhydrazyl assay on bark, fruits and leaf extracts of *C. grandicalyx*.



NEG, negative control; ASC, ; BB, bark boiled; BHW, bark hot water; BCW, bark cold water; LB, leaf boiled; LHW, leaf hot water; LCW, leaf cold water; FB, fruits boiled; FC, fruits chloroform; BA, bark acetone; BEA, bark ethyl acetate; BC, bark chloroform; BH, bark hexane; LA, leaf acetone; LEA, leaf ethyl acetate; LC, leaf chloroform; LH, leaf hexane; LCMP, Leave extracts compound.

Significant difference is represented by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for the negative control and values are mean ±SD of three replicates.

FIGURE 3: Radical scavenging of the different extracts of C. grandicalyx with ascorbic acid as the positive control.

Compound 1 appeared as a white amorphous powder whilst Compound 2 appeared white and oily. The compounds were subjected to NMR spectral analysis for structure elucidation.

Structure elucidation

Compound 1 was identified as 3β-hydroxylolean-12-ene (β-amyrin) with a chemical formula $C_{30}H_{50}O$, whilst Compound 2 was identified as 3β-acetoxyurs-12-ene (α-amyrin), with the chemical formula $C_{30}H_{50}O$. The compounds exhibited the same characteristic; however, Compound 2 revealed the presence of a methyl group on position 30 of the carbon ring. The spectral data results were similar to those reported previously (Table 2) (Dias et al. 2010; Menezes et al. 1998; Oliveira et al. 2010).

3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay

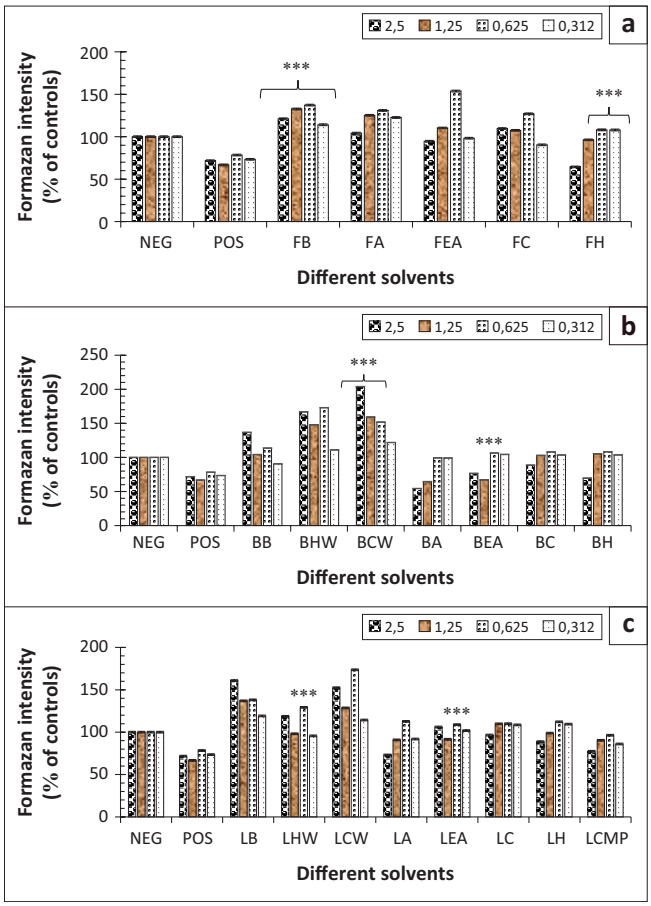
The cell viability was determined using MTT assay, based on the cellular conversion of tetrazolium salts by mitochondrial dehydrogenases of metabolically active cells to an insoluble purple formazan product. The assay was used to evaluate the cytotoxic effects of *C. grandicalyx* extracts on 3T3-L1 preadipocytes, C2C12 skeletal muscle, Vero and H-II-4E liver cells cultured *in vitro*. The viability of muscle cells treated with various extracts of *C. grandicalyx* was comparable to untreated

TABLE 2: ^{13}C NMR data of Compound 1 and 2 compared to ^{13}C NMR.

C	Compound 2	α-amyrin	Compound 1	β-amyrin
1	38.8	38.7	38.6	38.6
2	27.3	27.2	27.5	27.3
3	79.0	78.3	79.2	78.8
4	38.8	38.7	38.9	38.7
5	55.2	55.2	54.9	55.1
6	18.4	18.3	18.3	18.2
7	32.9	32.9	32.6	32.8
8	40.0	40.0	38.6	38.6
9	47.7	47.7	47.6	47.6
10	36.9	36.9	37.7	37.6
11	23.3	23.3	23.7	23.6
12	124.4	124.3	121.8	121.6
13	139.6	139.3	145.4	145.0
14	42.1	42.0	41.2	41.4
15	28.9	28.7	26.0	26.0
16	26.6	26.6	27.2	27.1
17	33.7	33.7	32.5	32.5
18	59.0	58.9	47.2	47.5
19	36.6	39.6	46.8	46.7
20	39.6	39.6	31.5	31.1
21	31.2	31.2	34.6	34.6
22	41.5	41.5	37.7	37.2
23	28.1	28.1	28.4	28.3
24	15.6	15.6	15.5	15.5
25	15.7	15.6	15.6	15.6
26	16.7	16.8	16.8	16.8
27	23.3	23.3	26.0	26.0
28	28.1	28.1	28.4	28.3
29	17.5	17.4	33.3	33.2
30	21.4	21.3	23.7	23.6

Source: From Menezes, F.D.S., Borsatto, Â.S. Pereira, N.A., De Abreu Matos, F.J. & Kaplan, M.A.C., 1998, 'Chamaedrydiol, an ursane triterpene from *Marsypianthes chamaedrys'*, *Phytochemistry* 48(2), 323. https://doi.org/10.1016/S0031-9422(97)01137-0

NMR, nuclear magnetic resonance.



NEG, negative control; POS, positive control; FB, fruits boiled; FA, fruits acetone; FEA, fruits ethyl acetate; FC, fruits chloroform; FH, fruits hexane; BB, bark boiled; BHW, bark hot water; BCW, bark cold water; BA, bark acetone; BEA, bark ethyl acetate; BC, bark chloroform; BH, bark hexane; LB, leaf boiled; LHW, leaf hot water; LCW, leaf cold water; LA, leaf acetone; LEA, leaf ethyl acetate; LC, leaf chloroform; LH, leaf hexane; LCMP, Leave extracts compound.

MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide.

Data presented mean ±SD of three replicates, *p*-significant of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

FIGURE 4: MTT assay on skeletal muscle (C2C12) cells treated with *C. grandicalyx* bark, leaves and fruits extracts for 24 h with H_2O_2 (5%) as a positive control (POS).

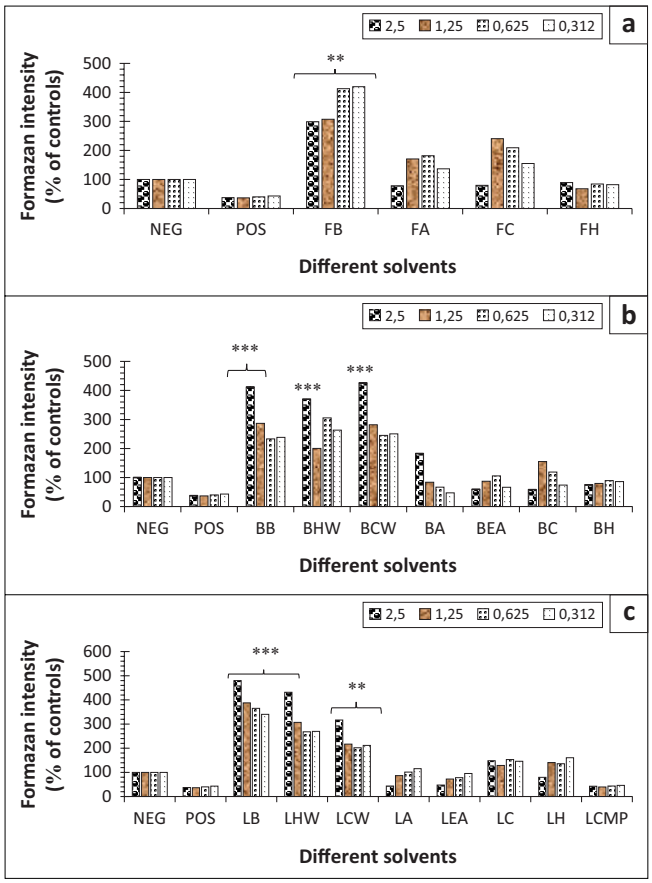
controls (Figure 4). The formazan intensity, an indicator of cell viability, was higher in muscle cells treated with bark, leaf and fruit extracts than untreated controls, suggesting that the plant extracts were not toxic to cells. Results obtained on 3T3-L1 cells revealed a similar trend as the muscle cells, with no toxicity observed in the different concentrations. The highest concentration (2.5 and 1.25 mg/mL) resulted in the highest increase in formazan intensity (Figure 5). FB, BB, BHW, BCW, LB, LHW and LCW extracts improved formazan intensity up to five-fold as compared to the negative control. LCMP proved to be toxic to the cells at all tested concentrations, yielding the same results as the positive control.

Similarly, the formazan intensity was higher in liver cells treated with extracts than untreated controls, suggesting that the plant extracts were not toxic to cells (Figure 6). Significant cell viability ($p < 0.001$) of about 150% was observed with some plant extracts (FB, BB, BHW and LB). Minimal toxicity was observed only at the highest concentration (2.5 mg/mL) for the BA, ethyl acetate and chloroform extracts (BC) but still below the lethal dosage 50 (LD_{50}). Lethal dose 50 refers to the standard concentration of a material that will kill half (50%) of the sample population

The Vero cells exhibited minimal toxicity at all concentrations (2.5, 1.25, 0.625, 0.312 mg/mL). In contrast, all other plant extracts demonstrated significant ($p < 0.001$) viability on the cells (Figure 7). The same pattern as with muscle, liver and preadipocytes was observed, with the highest concentrations (2.5 and 1.25 mg/mL) resulting in the highest formazan concentration. This finding may suggest that the plant extracts had mitogenic effects on cells and induced no adverse effect.

Discussions

No cytotoxicity was observed with all the cell lines, and the plant extracts exhibited an appreciable amount of phytochemical constituents which led to the isolation of two compounds. Cell viability indicates the biological status of the cells, including their cellular behaviour in response to treatments. The plant extracts were nontoxic to all the cells tested except for LCMP on preadipocytes; instead, they displayed increased viability. Viveros-Valdez et al. (2016) reported similar results on *C. boissieri*, where moderate toxicity was recorded. However, Jamkhande et al. (2013) reported potent activity against the brine shrimp by extracts of *C. verbenacea*. The high viability maintenance observed in the study is suggestive of the safety of the medicinal plant in traditional medicine.

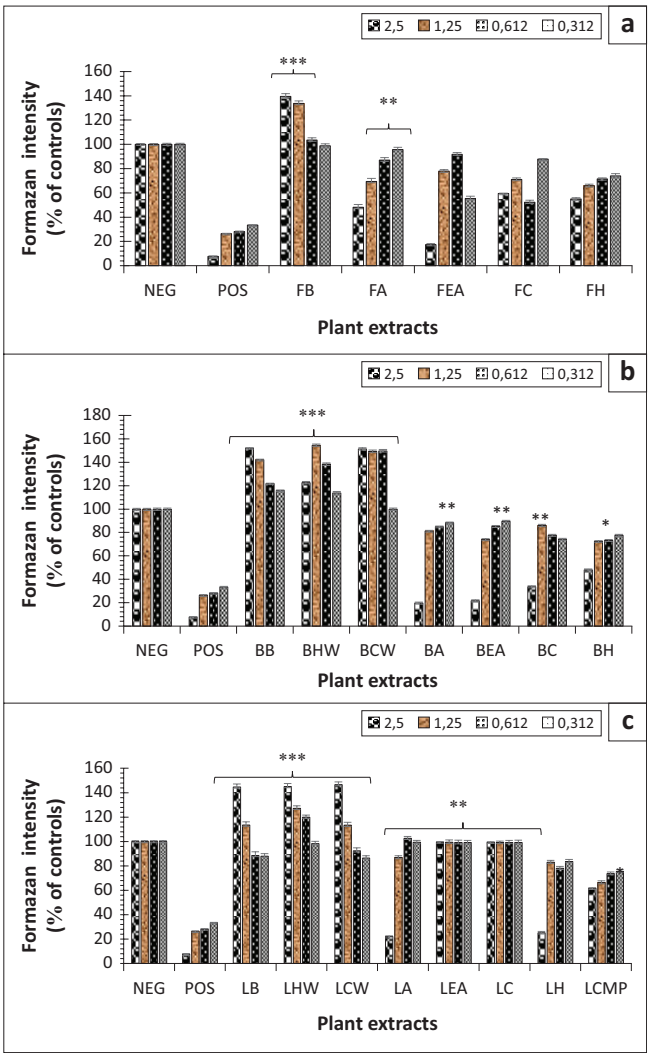


NEG, negative control; POS, positive control; FB, fruits boiled; FA, fruits acetone; FC, fruits chloroform; FH, fruits hexane; BB, bark boiled; BHW, bark hot water; BCW, bark cold water; BA, bark acetone; BEA, bark ethyl acetate; BC, bark chloroform; BH, bark hexane; LB, leaf boiled; LHW, leaf hot water; LCW, leaf cold water; LA, leaf acetone; LEA, leaf ethyl acetate; LC, leaf chloroform; LH, leaf hexane; LCMP, Leave extracts compound.

MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide.

Data presented mean ±SD of three replicates, *p*-significant of $*p < 0.05$, $**p < 0.01$ and $***p < 0.00$.

FIGURE 5: MTT assay on 3T3-L1 (preadipocytes) cells, cells treated with *C. grandicalyx* bark, leaves and fruits extracts for 24 h with H_2O_2 (50%) as a positive control.



NEG, negative control; POS, positive control; FB, fruits boiled; FA, fruits acetone; FEA, fruits ethyl acetate; FC, fruits chloroform; FH, fruits hexane; BB, bark boiled; BHW, bark hot water; BCW, bark cold water; BA, bark acetone; BEA, bark ethyl acetate; BC, bark chloroform; BH, bark hexane; LB, leaf boiled; LHW, leaf hot water; LCW, leaf cold water; LA, leaf acetone; LEA, leaf ethyl acetate; LC, leaf chloroform; LH, leaf hexane; LCMP, Leave extracts compound.

MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide.

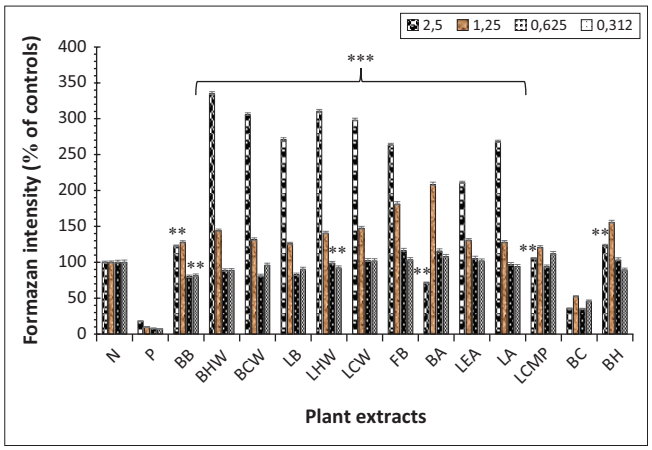
Values are mean ±SD of three replicates for the negative control.

FIGURE 6: MTT assay on the liver (H4IIE) cells treated with *C. grandicalyx* bark, leaves and fruits extracts for 24 h with hydrogen peroxide (50%) as a positive control (POS).

Amongst the extractants used in this study, polar extracts were the most effective. The antioxidant activity observed with the polar extracts, concur with the study by Masoko and Eloff (2007), who reported good antioxidant activity on the acetone and methanol extracts of Combretum and Terminalia species. Previous studies on species belonging to the genus *Cordia* also reported antioxidant activity (Kachhwaha & Gehlot 2015; Marini et al. 2018). Several *Cordia* species (*C. boissieri, C. dichotoma, C. globose and C. macleodii*) have been reported to possess antioxidant and anti-inflammatory activity (Sivakumar & Dhivya 2015). The amount of antioxidant activity of a plant depends on the effectiveness of the extracting solvent in dissolving the endogenous compounds (Kobus-Cisowska et al. 2020). Antioxidants are generally polar and thus can mostly be found in polar extracts than in nonpolar solvents (Maulidiani et al. 2018).

Antioxidants are important micronutrients because of their ability to inhibit or delay the progression of oxidative chain reactions and neutralise free radicals (Kacchhwaha & Gehlot 2019). Antioxidants provide resistance against oxidative stress through their scavenging activity, and the antioxidant activity of medicinal plants are often directly linked to phenolics, tannins and flavonoids contents (Marreiro et al. 2017; Novia et al. 2018). In addition, it is reported that antioxidant activity can be mainly ascribed to the presence of phenolic compounds (Sultana, Anwar & Przybylski 2007).

The amount of antioxidant activity observed can be attributed to the increased phenolic contents in polar extracts. Previous studies demonstrated that the extraction of phenolic compounds was affected by solvent polarity (Elfalleh, Kirkan & Sarikurkcu 2019). Phenolic compounds play a major role in oxidation because of their scavenging ability and may contribute directly to the antioxidant action (Ibrahim et al. 2019). Phenolic compounds are believed to be capable of acting on redox-sensitive signalling cascades and inhibit DNA damage, thereby increasing antioxidant activity (Genestra 2007). Phenolics, because of their different physical



NEG, negative control; POS, positive control; BB, bark boiled; BHW, bark hot water; BCW, bark cold water; LB, leaf boiled; LHW, leaf hot water; LCW, leaf cold water; FB, fruits boiled; BA, bark acetone; LEA, leaf ethyl acetate; LA, leaf acetone; LCMP, Leave extracts compound; BC, bark chloroform; BH, bark hexane.

MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide.

Significant differences are represented by *p < 0.05, **p <0.01 and ***p < 0.001 for the positive control and values are mean ±SD of three replicates.

FIGURE 7: MTT assay on Vero (Kidney) cells treated with *C. grandicalyx* bark, leaves and fruits extracts for 24 h with hydrogen peroxide (50%) as a positive control.

and chemical characteristics, are difficult to extract, and thus an appropriate method of extraction is yet to be developed (Dai & Mumper 2010).

Similarly, tannins were increased in polar extracts and absent in other solvents. The absence of tannins in hexane and other solvents might be attributed to the fact that tannins are easily extracted through infusion by water and not chemical methods (Ara et al. 2006). Furthermore, tannins are difficult to isolate in pure form; therefore, mixtures of polar, nonpolar and semipolar solvents like alcohol and acetone are normally used for extraction (Hari Prasath et al. 2017).

Likewise, alkaloids were predominant in the polar extracts. Alkaloids exist predominantly in the polar form with a small amount of nonpolar properties (Yadav, Bamotra & Tandon 2020). This could be the reason why they were present in most of the extracts, because they dissolve in neutral water and polar organic solvents such as chloroform and methanol. Generally, medicinal plants often contain a variety of alkaloids (Yubin et al. 2014), and they have been extensively studied because it is believed that they possess important biological properties such as antidiabetic, antioxidants and anti-inflammatory (Patel et al. 2016).

Other research works have reported some members of the genus as possible antioxidants (Mahasweta Roy et al. 2014). For instance, *C. dichotoma* and *C. retusa* exhibited an appreciable amount of phenolic acids, total phenolics and free radical scavenging activity (Kachhwaha & Gehlot 2015; Marini et al. 2018). In addition, the Boraginaceae family also reported the presence of pharmacologically active compounds such as naphthoquinones, flavonoids, terpenoids, phenols or purine derivatives (Dresler et al. 2016; Oza & Kulkarni 2017). Likewise, Abdel-Aleem et al. (2019) reported antioxidant, anti-inflammatory, analgesic, antipyretic and antidiabetic activities of *C. myxa* extract and fractions. There was a significant correlation between phenolics, flavonoids and antioxidant activity on the bark and leaf polar extracts.

The two isolated compounds were characterised using NMR spectrometry and identified as terpenoids, α-amyrin and β-amyrin. The spectral data of the compounds were similar to those previously reported (Dias et al. 2010; Menezes et al. 1998; Oliveira et al. 2010). They are designated α-amyrin because of the ursane skeleton and β-amyrin because of oleanane skeleton. Amyrins are three closely related natural chemical compounds, pentacyclic triterpenes with 30 carbon rings and the chemical formula $C_{30}H_{50}O$.

To date, phytochemical investigations of 36 species of the family resulted in the isolation of about 293 chemical constituents of different classes (Modak et al. 2007). Nazaruk and Borzym-Kluczyk (2015) reported triterpene compounds from the *Cordia* species. Amyrins are naturally distributed in plants and have been isolated from a variety of plant sources, such as the skin of tomatoes (Fernandez-Moreno et al. 2016). The three commonly known isolated pentacyclic amyrins are lupane, oleanane and ursane (Isah et al. 2016), and these have been indicated for the treatment of cancer (Laszczyk 2009).

To date, 48 triterpenoids from 11 species of *Cordia* have been reported (Oza & Kulkarni 2017), and a literature review has indicated that the majority of these terpenoids were isolated from the leaves (Matias et al. 2015). Pentacyclic triterpenes are often bioactive molecules possessing antioxidant, antitumour, antiviral, antidiabetic and anti-inflammatory activities with possible therapeutic potential (Jiao et al. 2019). This is not surprising because the two compounds isolated were from the leaves of *C. grandicalyx*. It has also been reported that several therapeutic effects on compounds from the Boraginaceae family used in ancient medicine are also applied in modern medicine (Dresler, Szymczak & Wójcik 2017; Gurib-Fakim et al. 2006).

Because of their ability to modulate the activity of several signalling networks, triterpenes have possible promising effects for the prevention and treatment of various pathological problems such as cardiovascular complications, inflammation, hepatotoxicity, tumour and cell proliferation (Nazaruk & Borzym-Kluczyk 2015). Inflammation is one of the major predisposing factors of diabetes mellitus, and it is not surprising that amyrins were extracted from *C. grandicalyx*, as it is indicated for the treatment of diabetes by traditional healers (Chauke et al. 2015). Santos et al. (2012) reported potential antihyperglycemic and hypolipidemic effects of α and β-amyrin mixture. The authors also indicated that these compounds could be used as possible lead

compounds for drug development and effective treatment of diabetes and atherosclerosis.

Therefore, the findings support the use of *C. grandicalyx* by traditional healers as an antidiabetic treatment, because the plant species contain compounds scientifically proven to have antidiabetic activity. The main limitation was that traditional healers use roots for the preparation of concoctions, but the aerial part was used because the roots could not be harvested because of deforestation. This, therefore, could have impacted the isolated compounds, because different compounds are found in different parts of the plant. The researchers therefore recommend that further studies be conducted on the roots to verify if similar compounds and activity would be observed.

Conclusion

Detection of active principles in medicinal plants is essential and may assist in understanding the nature of the compounds and their pharmacological effects. This study resulted in the isolation of two new compounds, α and β amyrins. To the best of the researchers' knowledge, no compound was isolated previously from *C. grandicalyx*. Both the bark and the leaf extracts had promising antioxidant activity and could therefore be considered a good source of natural antioxidants. No toxicity was observed with the plant extract against liver, Vero, 3T3 and skeletal muscle cells. The presence of the identified antioxidant activity and identified biologically active phytochemicals and compounds suggest that *C. grandicalyx* has potential pharmacological value, thereby supporting its use by traditional healers, albeit not as widespread.

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Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

Authors' contributions

M.A.C. carried out the experiment and wrote the manuscript with support from L.J.S., and L.K.M. L.J.S. and A.M.M. helped supervise the project and conceived the original idea, whilst L.K.M. analysed the spectra.

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Data availability

The data that support the findings of this study are available on request from the corresponding author, [M.A. Chauke]. The data is not publicly available because of the information that could compromise the study.

Disclaimer

The views expressed in this article are the authors' own and not those of Tshwane University of Technology or the NRF.

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