Isolation of lupeol from *Grewia lasiocarpa* stem bark: Antibacterial, antioxidant, and cytotoxicity activities

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Manuscript received: 27 July 2020. Revision accepted: 13 November 2020.

**Abstract.** Akwa N, Naidoo Y, Singh M, Thimmegowda SC, Nundkumar N, Lin J. 2020. Isolation of lupeol from *Grewia lasiocarpa* stem bark: Antibacterial, antioxidant, and cytotoxicity activities. Biodiversitas 21: 5684-5691. Lupeol a pentacyclic triterpenoid, was extracted from the stem bark of *Grewia lasiocarpa*. Stem bark was macerated with chloroform (CHCl₃) at 25 ± 2°C, and then purified using a silica gel column. The mobile phase was hexane (100%) and hexane: ethyl acetate (80:20) as the mobile phase. The structure of lupeol was identified by ¹H NMR, and ¹³C NMR spectral data, compared with reported data. The antibacterial, antioxidant and cytotoxicity activities of lupeol were evaluated using agar-well diffusion against six clinic bacteria isolates, namely *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 314588), and *Salmonella typhimurium* (ATCC 14026). The mobile phase was hexane (100%) and hexane: ethyl acetate (80:20) as the mobile phase. The structure of lupeol was identified by ¹H NMR, and ¹³C NMR spectral data, compared with reported data. The antibacterial, antioxidant and cytotoxicity activities of lupeol were evaluated using agar-well diffusion against six clinic bacteria isolates, namely *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 314588), and *Salmonella typhimurium* (ATCC 14026). Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC BAA-1683). The antioxidants assays were performed using 2,2-diphenyl-1-picyrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP). Cytotoxicity activity was carried out by MTT (3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide) assay was carried out against HEK293 (human embryonic kidney), HeLa (cervical carcinoma), and MCF-7 (breast adenocarcinoma) cell lines. The concentration range of lupeol was 240-15 µg/mL. Lupeol at the concentration of 240 and 120 µg/mL have no antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC BAA-1683). The antioxidant activity was dose-dependent, while cytotoxic activities were observed in HEK293, HeLa, and MCF-7 cells. This study was the first report on the extraction, isolation, and biological activities of lupeol, from the stem bark of *G. lasiocarpa*.

**Keywords:** Antibacterial, antioxidant, cytotoxicity, *Grewia lasiocarpa*, lupeol, stem bark

**INTRODUCTION**

*Grewia lasiocarpa* E. Mey. ex Harv. (Malvaceae) is a deciduous, fast-growing tropical shrub or small tree that usually grows on marginal soils (Boon and Pooley 2010). Species of this genus have been known to have numerous medicinal properties (Goyal 2012; Ullah et al. 2012; Sidhu and Zafar 2020) i.e., antibacterial, antimalarial, antifertility, antifungal, antioxidant, antiviral, antidiabetic, antipyretic, anti-inflammatory, anti-hemorrhagic, radioprotective, analgesic and hepatoprotective (Zia-Ul-Haq et al. 2012; Sinha et al. 2015; Shukla et al. 2016; Dwivedi and Manigauha 2017; Oribayo et al. 2018; Pramodini et al. 2018). However, *G. lasiocarpa* has been reported to have medicinal and nutritive properties (Akwu et al. 2019a, b, c).

The history of medicinal plant selection is usually based on the data of ethnopharmacology, ethnobotany, folkloric systems, or chemotaxonomic studies (Cordell 2015; Da et al. 2015; Alamgir 2017). These historical findings gave rise to phytochemistry, which is the study of the chemistry of phytochemical compounds. This study includes the direct isolation and identification of phytochemical compounds and their bioactivity evaluation (in vitro and in vivo) (Altemimi et al. 2017).

Globally, there has been a disproportionate increase in the rate of diseases/health problems and the number of new drugs being developed to combat diseases and resistance causative organisms (WHO 2011; Thirugnanasambandan and Kannayiram 2016; Weekes 2020). At present, there is a worldwide surge in the development of new drugs, particularly from medicinal plants (Altemimi et al. 2017; Ghosh et al. 2020). Physiological and biochemical processes in the human body are influenced by external and internal factors, which affect the production of free radicals and other reactive oxygen species. The external factors are exposure to industrial chemicals, radiations from ozone and x-rays, tobacco smoke, other air pollutants, while the internal factors involve the metabolic breakdown in the human body (Lobo et al. 2010; Juturu and Gormley 2013; Papadopoulou et al. 2020). Several advances in the study of free radicals and reactive oxygen species (ROS) have tremendously elevated the management of health and diseases because of their scavenging activity, i.e., cellular necrosis caused by auto-oxidation of cellular membrane lipids (Arung et al. 2018; Chen et al. 2018). Cancer is the leading disease in developed countries (Bajoriniene et al. 2019; Teoh et al. 2019).

Several alkaloids have been isolated from several *Grewia* spp., namely *G. bicolor*, *G. biloba*, *G. damine*, and *G. bilamellata*. These alkaloids are harman, 6-methoxyharman, 6-hydroxyharman, triterpenes (friedelin, epi-friedelan-3-ol), flavones (vitexin and isovitexin), neolignans (8-O-4’-neolignangauicacylglycerol-β-coniferyl ether (erythro), 8O4-neolignangauicacylglycerol-β-coniferyl ether...
ether(threo) (Jaspers et al. 1986, Jayasinghe et al. 2004, Ma et al. 2006). However, there is no information to date on the isolation, identification, and biological activities of any compound from the stem bark of G. lasiocarpa. Hence, our investigation was directed towards the isolation and identification of any compounds from the stem bark of G. lasiocarpa. Hence, our investigation was directed towards the isolation and identification of a chemical compound from the crude chloroform extract of G. lasiocarpa stem bark and investigate the antioxidant, antibacterial, and cytotoxicity activities of the isolated compound.

**MATERIALS AND METHODS**

**Plant materials**

The plant material (stem bark) of Grewia lasiocarpa was collected from the Umdoni Trust park, KwaZulu-Natal. Dr. Syd Ramdhanli carried out identification as a curator. A voucher specimen (Nnaka 002) was deposited in the Herbarium of the School of Life Sciences, University of KwaZulu-Natal, Durban, South Africa. The stem bark was dried, pulverized using a Waring blender, and stored in airtight amber storage before extraction.

**Extraction and isolation**

Approximately, 1100 g powder of G. lasiocarpa stem bark was subjected to a sequential extraction by increasing polarity using hexane (C₆H₁₄), chloroform (CHCl₃) and methanol (MeOH). The maceration was done at 25 ± 2°C at 160-200 rpm. The extracts were concentrated under reduced pressure using a rotary evaporator and stored in a drying room at room temperature.

**Column chromatography (CC)**

The isolation of the pure compound from the crude chloroform extract was carried out by column chromatography using silica gel 60 mesh size 70-230 (E. Merck, 0.063-0.200 mm) as a stationary phase, with gradient elution using n-hexane (100%) and n-hexane and ethyl acetate (80:20).

**Structural elucidation of the isolated compound**

The isolated compound was structurally elucidated by Nuclear Magnetic Resonance (NMR) spectroscopic analysis on a Bruker Avance™ 400 MHz spectrophotometer (Germany) at room temperature. About 25 mg of the pure compound was extracted with 700 µL of deuterated chloroform (CDCl₃) (Merck, Darmstadt, Germany). The 1D NMR (1H and 13C NMR) and DEPT, and 2D experiments (HSQC, HMBC, COSY, NOESY) were conducted. The NMR spectra were obtained under normal conditions and recorded at high resolution developed at 22°C using tetramethylsilane (TMS) as an internal reference.

**Gas Chromatography-Mass Spectrometry (GC-MS) analysis**

In a previous study, the crude chloroform extract of G. lasiocarpa stem bark was analyzed using gas chromatography unit (GCMS-QP2010 Plus Shimadzu) with a 30 m x 0.25 mm ID x 0.25 µm film thickness of a 5% phenyl methyl siloxane capillary column. The carrier gas was helium, and 2 µL of the extract was injected (splitless) with a column flow rate of 1.21 mL/min; Injection temperature and interface temperature were 240°C, and 220°C, respectively. The initial column temperature was 50°C held for 1.5 min, then increased to 300°C at a rate of 10°C/min, then held for 7 min, with a run time of 60 min within the scan range 40 to 500 m/z.

**Biological evaluation:** In vitro antioxidant, antibacterial, and cytotoxicity assays

**In vitro antibacterial assay**

The purified compound at the concentration range of 15-240 µg/mL was also evaluated for its ability to inhibit the growth of six pathogenic bacteria i.e., Gram-negative bacteria (Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Klebsiella pneumoniae (ATCC 314588) and Salmonella typhimurium (ATCC 14026); and Gram-positive bacteria: Staphylococcus aureus (ATCC 25923), methicillin-resistant Staphylococcus aureus (MRSA) (ATCC BAA-1683). The bacteria isolates were provided by Dr. Chunderika Mocktar, School of Pharmacy and Pharmacology, University of KwaZulu-Natal. The antibacterial assay was performed using the agar well diffusion method, with slight modifications (Perez et al., 1990). The inoculated Müller-Hinton agar plates were incubated at 37°C for 18-24 h, and the zones of inhibition were measured after the incubation period. Gentamicin and streptomycin were used as the positive controls at a concentration of 10 µg/mL for the Gram-negative and Gram-positive bacteria, respectively. The assay was performed in triplicate.

**In vitro antioxidant assay**

**DPPH free radical scavenging activity**

The hydrogen donating ability of the pure compound was determined using a modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) spectrophotometric assay as described by Braca et al. (2002). Briefly, 30 µL of the pure compound at a concentration range of 15-240 µg/mL was pipetted into a 96-well microplate in triplicate. After that, 150 µL of 0.3 mM DPPH solution was added into each well. The microplate was incubated in the dark at 25°C for 30 min. The absorbance of the pure compound was measured at 517 nm, and the percentage of the free radical inhibition was used to express the free radical scavenging activity. Ascorbic acid was used as the standard. The IC₅₀ was derived from the inhibition curves by plotting the percentage inhibition against the concentration logarithmic scale. The free radical scavenging ability of pure compound was calculated using equation 1:

\[
\text{DPPH scavenging activity (％)} = \left(\frac{\text{Abs}_\text{control} - \text{Abs}_\text{sample}}{\text{Abs}_\text{control}}\right) \times 100 \quad (1)
\]

Where:

Abs<sub>control</sub> is the absorbance of DPPH and methanol

Abs<sub>sample</sub> is the absorbance of DPPH radical + sample (compound or standard)
Ferric reducing antioxidant power (FRAP) assay.
The ferric reducing power of the pure compound was determined using a modified FRAP assay as described by Benzie and Strain (1996). Briefly, 50 µL of 0.2 M sodium phosphate buffer (pH 6.6) and 100 µL of 1% potassium ferricyanide were added to 50 µL of the pure compound at a concentration range of 15-240 µg/mL. The microplate was incubated at 50°C for 30 min. After incubation, each well was added with 50 µL of 10% trichloroacetic acid, 50 µL of distilled water and 10 µL of 0.1% iron (III) chloride ([FeCl₃]), and homogenized. Thereafter, 200 µL of the homogenized solution was transferred to a 96 well microplate, and the absorbance was measured at 700 nm. The results were expressed as a percentage of the absorbance of the crude extracts to that of gallic acid using the formula (equation 2):

\[
% \text{ inhibition} = \frac{\text{Abs of sample} - \text{Abs of garlic acid}}{\text{Abs of garlic acid}} \times 100
\]

Where: Abs: absorbance

Cell viability assessment by MTT assay
The MTT cell viability assay using the HEK293, HeLa, and MCF-7 cells was carried out as described by Mosmann (1983). The HEK293 (human embryonic kidney) cells were obtained from The University of Witwatersrand Medical School, South Africa, HeLa (cervical carcinoma) and MCF-7 (breast adenocarcinoma) cells were purchased from ATCC, Manassas, USA.

The cells were trypsinized upon 90% confluency and seeded into a 48-well plate at a density of 3 x 10⁴ cells/well. The cells were incubated at 37°C for 24 h for attachment. After the incubation, the media was changed, and the different concentrations of compound (15-240 µg/mL) was added to the wells. The plates were incubated again for 48 h at 37°C. After that, the media was changed, and 200 µL of fresh medium containing 20 µL of MTT (5 mg/mL in phosphate-buffered saline (PBS)) was added and cells incubated for four hours. The media containing the MTT reagent was then removed and 200 µL DMSO was quickly added to solubilize the formazan crystals. The absorbance was read at 540 nm using a Mindray MR-96A microplate reader (Vacutec, Hamburg, Germany). All assays were performed in triplicate, using untreated cells as a control.

Statistical analysis
The data were analyzed using EXCEL and SPSS 25 for Windows, IBM Corporation, New York, USA. Data obtained were expressed as mean ± standard deviation. P-values of <0.05 were considered significant. All analyses were performed in triplicate. (n=3).

RESULTS AND DISCUSSION
Lupeol is usually found in fruit waxes, leaves, and stem bark (Sánchez-Burgos et al. 2015; Vithana et al. 2019; Jahan and Makbul 2020). It has been isolated from the stem of *Grewia asiatica* (Chattopadhyay and Pakrashi 1975), stem bark of *Grewia optiva* (Ud din et al. 2013), whole plant of *Grewia bicolor* (Jaspers et al. 1986), the root bark of *Grewia rothi* (Bhagat and Misra 1974), roots and stem bark of *Grewia tiliae folia* (Badami et al. 2004).

A previous study revealed that lupeol was present in the crude stem bark chloroform extract of *G. lasiocarpa* (Akwu et al. 2019c) with a retention time of lupeol (C₃₀H₄₂O), as evidenced by a molecular ion peak m/z 426 [M]⁺ was 29.20 min (Figure 1A). However, Soujanya et al. (2017) reported a retention time of 27.16, 28.57, and 28.15. The NMR spectral ¹H NMR spectrum and ¹³CNMR (Figures 1B and 1C) respectively, revealed that the isolated compound is lupeol with the chemical structure as depicted in Figure 2.E (Silva 2017; Beserra et al. 2018, Gurupriya et al. 2018; Balde et al. 2019). The NMR spectroscopic, structural analyses confirmed that the isolated compound (I) is lupeol (Figures 1A, 1B, and 2A-D) and the chemical structure, as illustrated in Figure 2.E. Compound (I): white powder, ¹H NMR (CDCl₃, 400 MHz) δ 4.57 (s, H-29a), 4.68(s, H-29b), 3.20 (1H, dd, J= 5.12, 11.27 Hz, H-3), 1.68, 1.02, 0.98, 0.96, 0.82, 0.76, 0.70 (21 H, 7 s, 7 CH₃) (Figure 2). ¹³C NMR (CDCl₃, 400 MHz) δ: 38.7 (C-1), 27.3 (C-2), 79.0 (C-3), 38.7 (C-4), 55.3 (C-5), 18.3 (C-6), 34.2 (C-7), 40.9 (C-8), 50.4 (C-9), 37.1 (C-10), 20.8 (C-11), 25.23 (C-12), 38.8 (C-13), 42.7 (C-14), 27.4 (C-15), 34.2 (C-16), 42.7 (C-17), 48.3 (C-18), 48.7 (C-19), 150.4 (C-20), 29.7 (C-21), 40.9 (C-22), 27.9 (C-23), 15.3 (C-24), 16.1 (C-25), 15.9 (C-26), 14.7 (C-27), 18.3 (C-28), 109.6 (C-29), 19.0 (C-30). It could be proposed that the molecular formula is C₃₀H₄₂O. The ¹H NMR spectrum showed seven tertiary methyl singlets at δ 0.70, 0.76, 0.82, 0.96, 0.98, 1.02, 1.68, and one secondary hydroxyl group as a doublet of doublets at δ 3.20. It also showed two olefinic protons at δ 4.57 and 4.68 representing the exocyclic double bond as in 1. The ¹³C NMR spectrum of the compound showed 30 signals for the terpenoid skeleton, which included a carbon bonded to the oxygen bond as in 1. The NMR spectral data have been reported (Jain and Bari 2010; Moradkhani et al. 2016). Lupeol has been reported to possess antioxidant, antimicrobial, anti-tumor, and anti-carcinogenic activities (Wal et al. 2011; Ogunlaja 2017; Kumari et al. 2018; Swargiary et al. 2020).

A previous study by Amoussa et al. (2016) showed that lupeol at 100 µg/disc was not effective in inhibiting the growth of *Pseudomonas aeruginosa*: *Staphylococcus epidermidis*: *Candida albicans*, and *Enterococcus faecalis*. The antibacterial activity result of this study (Table 1) showed that *Pseudomonas aeruginosa* was resistant to lupeol. A study by Patel et al. (2018) showed that lupeol inhibits the growth of *P. aeruginosa* at concentrations of 25-5 µg/mL. However, this study showed that only methicillin-resistant *Staphylococcus aureus* (MRSA) was insignificantly susceptible to lupeol compared to positive control (Table 1). This insignificant activity was following the result by Gallo and Sarachine (2009). The disparities in the inhibition against *P. aeruginosa* from this study, Amoussa et al. (2016) and Patel et al. (2018) may be attributed to the genetic differences that may exist between bacteria strains of the same genus and species.
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Figure 1A. GC-MS chromatograph of lupeol

Figure 1B. $^1$H NMR spectrum of lupeol in CDCl$_3$.

Figure 1C. $^{13}$CNMR spectrum of lupeol in CDCl$_3$.

Figure 2A. HMBC (CDCl$_3$, 400 MHz) of compound (1) lupeol isolated from the stem bark of Grewia lasiocarpa.

Figure 2B. NOESY (CDCl$_3$, 400 MHz) of compound (1) lupeol isolated from the stem bark of Grewia lasiocarpa.

Figure 2C. COSY (CDCl$_3$, 400 MHz) of compound (1) lupeol isolated from the stem bark of Grewia lasiocarpa.

Figure 2D. HSQC (CDCl$_3$, 400 MHz) of compound (1) lupeol isolated from the stem bark of Grewia lasiocarpa.

Figure 2E. Chemical structure of lupeol.
**Figure 3.A.** DPPH Free radical scavenging activity of compound (1) isolated from the stem bark of *Grewia lasiocarpa* at various concentrations. *a* and *b* letters above the bars for a given concentration are significantly different between treatment (Tukey’s honest significant difference multiple range post hoc test \( p<0.05 \) IBM SPSS version 25). GL: *Grewia lasiocarpa*, AA: Ascorbic acid, compd.1: compound.

**Figure 3.B.** Ferric Reducing Antioxidant Power values of compound (1) isolated from the stem bark of *Grewia lasiocarpa* at various concentrations. *a*-*b* letters above the bars for a given concentration are significantly different between treatment other (Tukey’s honest significant difference multiple range post hoc test \( p<0.05 \) IBM SPSS version 25). GL: *Grewia lasiocarpa*, GA: gallic acid.

**Figure 4.** Effect of lupeol on viability on HEK293, HeLa and MCF-7 cell lines (MTT cell viability assay). Data is represented as mean ± SD (\( n=3 \)). *a* letter above the bar for a given concentration is not significantly different from each other (Tukey’s honest significant difference multiple range post hoc test \( p<0.05 \) IBM SPSS version 25).

**Table 1.** Antibacterial activity of the pure compound (1) extracted from *Grewia lasiocarpa* stem bark (zone of inhibition mm).

| Bacterial strains | Concentration (µg/mL) | 10 µg/mL (positive control) |
|-------------------|-----------------------|-----------------------------|
|                   | 240 | 120 | 60 | 30 | 15 | R          |
| MRSA              | 6.50 | 6.50 | 6.50 | 6.50 | R    | 12.33 ± 2.31* |
| SA                | R   | R   | R   | R   | R    | 7.75 ± 0.00*  |
| PA                | R   | R   | R   | R   | R    | 13.55 ± 3.24 |
| ST                | R   | R   | R   | R   | R    | 19.33 ± 1.92  |
| KP                | R   | R   | R   | R   | R    | 14.22 ± 3.77  |
| EC                | R   | R   | R   | R   | R    | 18.33 ± 1.68  |

Note: R: resistant, SA: *Staphylococcus aureus* (ATCC 25923), Meticillin-resistant *Staphylococcus aureus* [MRSA] (ATCC BAA-1683), PA: *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14026), KP: *Klebsiella pneumoniae* (ATCC 314588), EC: *Escherichia coli* (ATCC 25922), Positive control: Streptomycin (10 µg/mL*); Gentamicin (10 µg/mL) and (\( n = 3 \)), N/A: Not applicable.

**Table 2.** IC\(_{50}\) values of compound (1) isolated from the stem bark of *Grewia lasiocarpa*, ascorbic acid (AA), and gallic acid (GA) used for the DPPH and FRAP assays.

| Sample  | DPPH (µg/mL) | FRAP (µg/mL) |
|---------|--------------|--------------|
| AA      | 65.09±1.81   | -            |
| GA      | -            | 51.12 ± 1.71 |
| Compound 1 | >1000        | >1000        |

Note: The IC\(_{50}\) of the DPPH and FRAP assay of compound (1) isolated from the crude chloroform extract of the stem bark of *Grewia lasiocarpa* E. Mey. ex Harv. Data are presented as mean, \( n=3 \), of triplicate determinations. AA: ascorbic acid, GA: gallic acid.
Table 3. IC50 values of lupeol on HEK293, HeLa and MCF-7 cell lines

| Cell lines | IC50 value (mg/mL) |
|------------|--------------------|
| HEK293     | ND                 |
| HeLa       | >1000              |
| MCF-7      | >1000              |

The results showed that the inhibition percentage of the ascorbic acid against free radicals DPPH was dose-dependent; however, the inhibition percentage of compound (1) was not dose-dependent (Figure 3A). The results of FRAP also have a similar pattern (Figure 3B). The IC50 of ascorbic acid and gallic acid was 65.09 ± 1.81 µg/mL and 51.12 ± 1.71 µg/mL for DPPH and FRAP assays, respectively. The IC50 value of compound (1) was >1000 µg/mL (Table 2). This result was similar to Tchimene et al. (2016) that lupeol exhibited a more potent antioxidant activity at 800 µg/mL, implying that if a higher concentration were used, the IC50 value would have been lower (Table 2). Thus, lupeol does not have a strong antioxidant activity at low concentrations. Besides, the application of other antioxidant assays such as ferrous oxidation-xylene orange (FOX), β-carotene bleaching assay, 2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), butylated hydroxytoluene (BHT), and ferric thiocyanate (FTC) assays might have resulted in a better antioxidant activity as acknowledged by Can-Cauich et al. (2019). This observation, therefore, suggests at least three antioxidant activity assays should be conducted to determine the antioxidant activity of a compound.

Compound (1) lupeol, exhibited dose-dependent cytotoxicity on two of the mammalian cancer cell lines used (Figure 4) which was similar to that reported by Lambertini et al. (2005) and Saleem (2009). Lupeol exhibited no significant cytotoxic effect on the HeLa and MCF-7 cell lines used, with IC50 values greater than 1000 µg/mL (Table 3). Lupeol has cytotoxic activity against the HEK293 cell line; however, the IC50 of HEK293 was not determined because the cytotoxic activity exhibited was not dose-dependent (Table 3). The IC50 values > 1000 µg/mL of the antioxidant and cytotoxicity assays are suggestive that lupeol may exhibit weak activities at low concentrations.

In conclusion, we have confirmed and reported for the first time the presence of lupeol in the stem bark of *Grewia lasiocarpa*. Lupeol has indicated to have antibacterial and antioxidant activities, which was concentration-dependent. The results from the MTT cytotoxicity studies revealed that lupeol caused cell death in the HEK293, HeLa, and MCF-7 cells. However, the mechanism of cell death (apoptosis, etc.), still needs to be fully elucidated.

ACKNOWLEDGEMENTS

The authors would like to gratefully acknowledge the Organisation for Women in Science for the Developing World (OWSD) and Swedish International Development Cooperation Agency (SIDA) for their financial and intellectual contributions. The National Research Foundation (NRF) for the provision of consumables. Dr. Mrs. E.A. Ofusori, Dr. S.O. Bode, Dr. N. Mahlangeni, Dr. K. Nagaraju, S. Shange, Dr. Nisar, and Mrs. F.O. Joshua for their assistance with the structural elucidation.

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