

# Phytochemical analysis and antibacterial evaluation of the ethyl acetate extract of the stem bark of *Bridelia micrantha*

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Submitted: 31-01-2012

Revised: 16-03-2012

Published: 05-03-2013

## ABSTRACT

**Background:** Plant cells fundamentally are chemical factories containing a rich supply of therapeutically useful phytochemicals that have the potential of being developed into potent antimicrobial agents. **Aim of the Study:** To investigate the antibacterial activity of fractionated extracts of the ethyl acetate extract of the stem bark of *Bridelia micrantha* (Hochst., Baill., Euphorbiaceae). **Materials and Methods:** Thin-layer chromatography and column chromatography were used to purify the extracts and antimicrobial activity performed on reference and clinical strains of *Staphylococcus aureus*, *Shigella sonnei*, *Salmonella Typhimurium*, and *Helicobacter pylori* using direct and indirect bioautographic methods respectively. Furthermore, the eluted compound fractions were then assayed for minimum inhibitory concentration (MIC<sub>50</sub>) using the 96-well micro dilution technique. **Results:** Better separation of phytochemicals was obtained from the non-polar Benzene/Ethanol/Ammonia (BEA) and intermediate-polar Chloroform/Ethyl acetate/Formic acid (CEF) eluents compared to the polar Ethanol/Methanol/Water (EMW). Bioautography revealed the presence of three bioactive compounds (R<sub>f</sub> values; 0.12, 0.20, and 0.42) on the BEA plates, designated fractions 3, 7, and 8 with MIC<sub>50</sub> values; 0.0048mg/mL to 1.25mg/mL (fraction 3), 0.0024mg/mL to 5 mg/mL (fraction 7), and 0.0024mg/mL to 2.5mg/mL (fraction 8). **Conclusion:** Our findings demonstrate that ethyl acetate extract of the stem-bark of *B. micrantha* possess potent bioactive phytochemicals that may be developed into new antimicrobials.

**Key words:** Antibacterial activity, *Bridelia micrantha*, euphorbiaceae, medicinal plants, minimum inhibitory concentration,

## INTRODUCTION

Several hundreds of plants the world over represent good sources of therapeutic agents and are used traditionally for different purposes, including treatment of bacteria, fungi, and viral infections.<sup>[1,2]</sup> Of about 250,000 flowering plants in the world<sup>[3]</sup> more than 50,000 are used for medicinal purposes.<sup>[4]</sup>

Drug discovery from medicinal plants led to the isolation of anti-infectives such as vincristine, vinblastine, aspirin, cocaine, digitoxin, quinine and morphine, of which some are still in use.<sup>[5]</sup> Morphine isolated from *Papaver somniferum*

is used as a potent pain killer, while aspirin, an acetyl salt of salicylic acid from *Willow* bark, is one of the most effective analgesic, antipyretic, and anti-inflammatory agent used in modern day medicine.<sup>[5,6]</sup> Cocaine from coca plant, *Erythroxylum coca*, has been used as substrate in the synthesis of local anaesthetic agents such procaine and xylocaine, while quinine from *Cinchona* bark is used in the management of severe/cerebral malaria in sub-Saharan Africa.<sup>[5]</sup>

The use of medicinal plants in the treatment of diseases in Africa is an ancient tradition that has co-existed with human habitation.<sup>[7]</sup> Herbal medicines form a significant part of African culture and traditions,<sup>[8,9]</sup> hence there is an increasing trend to integrate traditional medicine with primary health care. This owing to the fact that 80% of families in the developing world today still depend on traditional herbal remedies for the treatment of disease, as they are affordable and easily accessible to all.<sup>[7]</sup>

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### Access this article online

#### Website:

www.phcog.com

#### DOI:

10.4103/0973-1296.108139

#### Quick Response Code:



*Bridellia micrantha* belongs to the genus *Bridellia* Willd (Tribe; Brideliaceae, Phyllanthaceae, Order; Malpighiales)<sup>[10]</sup> and includes approximately 60-70 species from Africa to Asia. The plant is a semi-deciduous to deciduous tree up to 20 m tall with a dense rounded crown and tall, bare stem. Several parts of the plant are used to treat different human ailments by traditional healers. The leaf sap of the plant is used to treat sore eyes by the Hayas; the shambalas use the roots as a remedy for severe epigastric pain while the Zigulas rub a preparation of the powdered root, made with oil or butter into the scalp for the relief of headache.<sup>[11]</sup>

*S. aureus*, *S. sonnei*, *S. Typhimurium*, and *H. pylori* are pathogens of medical importance causing life threatening infections such as; meningitis, shigellosis, non-typhoidal salmonellosis, and peptic ulcer<sup>[12]</sup> which are difficult to treat due to the emergence of multidrug resistant strains (MRSA, VRSA, and *S. Typhimurium* DT104).<sup>[13]</sup>

In South Africa, *B. micrantha* stem bark is used in traditional medicine for gastrointestinal ailments, paralysis and painful joints<sup>[14]</sup> while in five districts of Lagos, Nigeria the stem bark is used for the treatment of diabetes.<sup>[15]</sup> In the Sango bay ecosystem in Rakai district, central Uganda, decoction of the bark and leaves is used for treating syphilis and the bark for pre-hepatic jaundice.<sup>[16]</sup> Crude methanol extract of *B. micrantha* has been found to be active against the RNA-dependent DNA polymerization (RDDP) function of HIV-1 reverse transcriptase enzyme.<sup>[1]</sup> Studies have also shown that extracts of the whole stem demonstrate antimicrobial activity by inhibiting the growth of *Campylobacter jejuni/coli*.<sup>[17]</sup> However, their effect on drug resistant Gram-positive and Gram-negative pathogens which commonly circulate in our environment has not been investigated.

Recent studies have shown that *B. micrantha* root, stem bark and leaf constitutes various phytochemical compounds such as; friedelin, epi-friedelin, flavonoids, tannins, gallic acid, ellagic acid, sterol, saponin, anthocyanidin, delphinidin, caffeic acid, taraxerone and taraxerol some of which possess potent antimicrobial activity.<sup>[1,18]</sup> In this study we report on the antibacterial activity of fractionated extracts of the stem bark of *B. micrantha*.

## MATERIALS AND METHODS

### Bacterial species

Bacterial species used in this study include; reference strains of *S. aureus* NCTC 6571, *S. Typhimurium* ATCC 13311, *S. sonnei* ATCC 29930, and *H. pylori* 252c (a local drug resistant strain isolated from gastric biopsy in our laboratory).<sup>[19]</sup> These strains were selected based on the frequency of drug resistance they exhibit in the developing world.<sup>[20]</sup>

### Preparation of plant extract

The stem bark of *B. micrantha* was selected based on ethnobotanical information and was identified in collaboration with botanists at the University of Venda, Limpopo Province, South Africa where voucher specimens (BPO3) have been deposited.

The method described by Ndip *et al.*,<sup>[21]</sup> was used with modifications. The harvested plant was air dried for 2 weeks and ground to fine powder using a blender (ATO MSE mix, 702732, England). Ethyl acetate was used for extraction. Dried plant material, 2.5 kg, was macerated in fivefold excess of the solvent in extraction bottle such that the level of the solvent was above that of the plant material. The mixture was placed in a shaker (Edison, N.J., USA) at room temperature (RT) for 48hrs and then centrifuged (Model TJ-6 Beckman, USA) at 3000 rpm for 5 mins, and later filtered using filter paper of pore size 60 Å. This process was repeated twice for a total of three extractions (exhaustive extraction). The combined filtrate was concentrated in a rotavapor (BUCHI R461, Switzerland) and the plant extract obtained was transferred to a vial and allowed to stand at room temperature for 24hrs to permit evaporation of the residual solvent. Stock solution was prepared by dissolving three grams of the extract in 10% Dimethyl Sulphoxide (DMSO) and the remainder kept in the extract bank.

### Phytochemical analysis

Phytochemical constituents of the plant extract (ethyl acetate extract) was analyzed by thin layer chromatography (TLC) as described by Ellof *et al.*,<sup>[22]</sup> using aluminium-backed TLC plates (MERCK, silica gel 60 F<sub>254</sub> plates). Briefly, the plant extract was reconstituted in the extracting solvent (i.e. ethyl acetate) to 13mg/mL, 11mg/mL, 9mg/mL, 7mg/mL, and 5mg/mL concentrations respectively. Five microlitres of each extract concentration was spotted on the TLC plates (2cm from one end of the plate) and developed in different mobile systems (eluent): ethyl acetate/methanol/water (40mL: 5.4mL: 4mL) [EMW], chloroform/ethyl acetate/formic acid (32mL: 4mL: 4mL) [CEF], and benzene/ethanol/ammonium hydroxide (36mL: 4mL: 0.4mL) [BEA] respectively.<sup>[23]</sup> Development of the chromatograms was done in a closed tank in which the atmosphere had been saturated with the eluent vapour. The plates were then dried overnight under a stream of air to remove excess solvent.

Visible bands were viewed under daylight and under UV light at 302 nm and 365 nm respectively before being sprayed with a freshly prepared Vanillin reagent (0.1 g vanillin powder + 14mL of methanol + 0.5mL sulphuric acid). The plates were carefully heated at 105°C for optimal colour development.<sup>[24]</sup>

### Retention factor ( $R_f$ ) values of compounds

Characterization of the different compounds identified was done by calculating their  $R_f$  values in the different TLC system.<sup>[25]</sup> Briefly, the solvent front was marked on the TLC plates immediately after removing it from the chamber, and allowed to dry before visualizing the bands relating to different compounds. The  $R_f$  value was calculated by the following equation:

$$R_f \text{ value} = \frac{\text{distance moved by the component from the origin to spot centre.}}{\text{distance moved from origin to solvent front.}}$$

### Antimicrobial assay by bioautography

The bioautography procedure was done according to the modified method of Masoko *et al.* Eloff.<sup>[26]</sup> TLC plates (5 × 15cm) were loaded with 100 and 200µg (5µL of 20 and 40mg/mL) of the extract. The prepared plates were developed in the three different mobile systems (CEF, BEA and EMW) separately. The chromatograms were dried for up to a week at room temperature under a stream of air to remove remaining solvent. Cultures (*S. aureus*, *S. sonnei*, *S. Typhimurium*) were grown on nutrient agar at 37°C for 24hrs. BHI broth was prepared in 50mL bottles and the cultures transferred into the broth from agar plates with sterile swabs. The TLC plates developed were inoculated with a fine spray of the concentrated suspension containing approximately 10<sup>9</sup> actively growing bacteria per mL. The plates were sprayed until they were just wet, incubated overnight at 37°C in a clean bioautography chamber with 100% relative humidity and then sprayed with a 2 mg/mL solution of piodonitrotetrazolium violet (INT) (Sigma®) (0.2 g INT powder + 1mL methanol + 99mL water) and further incubated for 30 min to 1hr at 37°C. White areas indicated the spots where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the tested bacteria.

Bioautography for *H. pylori* was performed using the indirect method (agar overlay).<sup>[26]</sup> Briefly, after developing the TLC plate as described above, it was placed in a sterile petri dish. A freshly prepared BHI agar (Oxoid, England) supplemented with 7% horse blood (Oxoid, England) and Skirrow's supplement (Oxoid, England) was poured directly on the TLC plate in the petri dish. This was then inoculated with *H. pylori* and incubated microaerobically at 37°C for 3 days. Inhibition of *H. pylori* growth was seen as a clear zone on the plate.

### Column chromatography

The fractions with antibacterial activity were subjected to Column chromatography for purification. Silica gel was used as the stationary phase (absorbent) while a mixture

of benzene/ethanol/ammonium hydroxide in the volume ratio used in TLC was used as the mobile phase (eluent).<sup>[27]</sup>

Briefly, 6gm of plant extract was added to 12gm of silica gel and ground using a ceramic mortar and pestle to a fine powdered form. This was then added to a packed column (silica gel packed with benzene which is the least polar in the solvent mixture). The mobile phase (benzene/ethanol/ammonium hydroxide) was continuously poured to the top with the aid of a dropper. The bottom outlet of the column was opened, allowing the eluent to flow through the column. As the eluent passed down the column, the compound fraction moved down the column. The separated fraction flowed out of the column where the different eluates were collected in separate bottles.<sup>[27]</sup>

### Determination of minimum inhibitory concentration (MIC<sub>50</sub>) of eluted fractions

The MIC was determined using the 96-well micro dilution method as described by Banfi *et al.*<sup>[28]</sup> with modification. All the fractions eluted from column chromatography were used. Two-fold dilutions of the eluted fractions were prepared in the test wells in complete BHI broth (Oxoid, England), the final extract concentrations being 0.0024mg/mL-5mg/mL. Each bacterial strain were sub cultured in 5mL of BHI broth for 3 days (*H. pylori*), and 24hrs for the other organisms. Twenty micro litres of each bacterial suspension was added to 180µL of fraction-containing culture medium. Control wells were prepared with culture medium and bacterial suspension only. The plates were sealed and incubated under microaerophilic condition for 3–5 days (*H. pylori*), and aerobically for 24–48hrs (*S. sonnei*, *S. aureus*, *S. Typhimurium*) at 37°C. After each incubation period, 20µL of resazurin solution was added per well, colouring them blue. Plates were then incubated at 37°C for an additional hour, after which the plates were observed for colour change from blue to pink in live microorganism-containing wells and then read with a micro titre plate reader (Model 680 Bio-Rad, Japan) adjusted to 620nm. MIC<sub>50</sub> was then determined as the lowest fraction concentration that inhibited bacterial growth by 50%. Ciprofloxacin was included as a positive control.

## RESULTS AND DISCUSSION

### Thin layer chromatography analysis of ethyl acetate extract of *B. micrantha*

In a separate study, we established that the stem bark of *B. micrantha* demonstrated antimicrobial activity against the test organisms.<sup>[29]</sup> In this study, the TLC chromatograms were developed in three eluent systems of different polarity, EMW (polar), CEF (intermediate polar), and BEA (non-polar).



The TLC chromatograms of the ethyl acetate extract are shown in Figures 1a and b. Comparing the compound profiles, TLC chromatogram developed in EMW showed 3 compounds under UV light (wavelength 302 nm), 1 compound under UV light (wavelength 365 nm), and 4 compounds after spraying with vanillin reagent. Chromatogram developed in the CEF system showed, 4 compounds under UV light (wavelength 302 nm), 2 compounds under UV light (wavelength 365 nm), and 11 compounds after spraying with vanillin reagent. For chromatogram developed in BEA, 4 compounds were visualized under UV light (wavelength 302 nm), 1 compound under UV light (wavelength 365 nm), and 12 compounds after spraying with vanillin reagent. On comparing the  $R_f$  values of the compounds seen under UV light (wavelengths 302 nm & 365 nm) with those seen after spraying with vanillin, it was observed that all the compounds seen under UV light were also visible after spraying with vanillin reagent; though, some compounds quickly disappeared after oxidation on exposure to air [Table 1].

Plant cells may possess a rich supply of therapeutically useful constituents.<sup>[30]</sup> Phytochemical analysis of the ethyl acetate extract of *B. micrantha* showed that the non-polar BEA system was efficient in the purification process giving a much more separated profile of compounds (12), while the polar EMW system did not perform well in the process giving just 4 compounds as revealed by the band profiles. The lack of adequately separated compounds on the EMW

system is probably because most of the compounds were relatively non-polar.<sup>[24]</sup>

#### Antimicrobial assay by bioautography

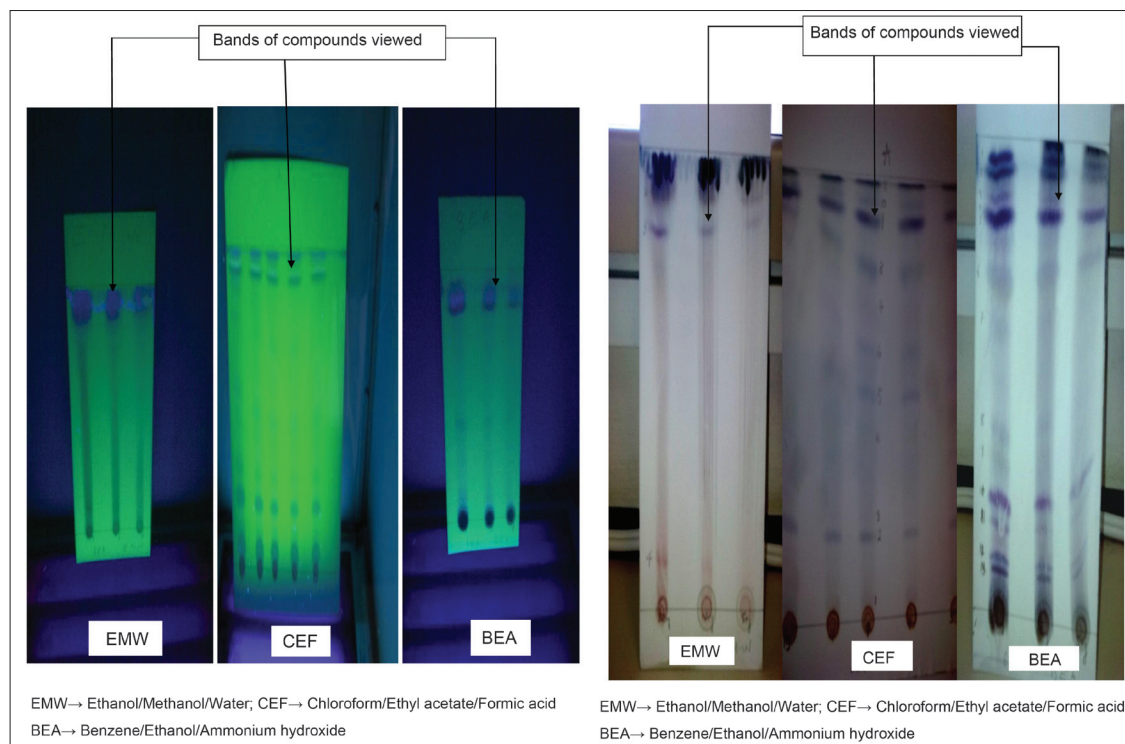
Bioautography was performed on TLC chromatogram developed in the BEA (non-polar) and CEF (intermediate polar) eluent system because they separated more compounds compared to the EMW eluent system. Inhibition zones were observed as white spots on a purple-red background on the TLC plates. Scoring was used to determine relative activity of the bioactive compounds [Table 2].

Bioautography on TLC chromatogram developed in the

**Table 1:  $R_f$  value of visualized compounds on thin layer chromatography plates**

Compound	EMW	CEF	BEA
Compound 1	0.97	0.98	0.97
Compound 2	0.91	0.94	0.93
Compound 3	0.84	0.90	0.87
Compound 4	0.11	0.80	0.85
Compound 5	–	0.72	0.73
Compound 6	–	0.60	0.63
Compound 7	–	0.51	0.42
Compound 8	–	0.42	0.36
Compound 9	–	0.24	0.26
Compound 10	–	0.19	0.20
Compound 11	–	0.06	0.12
Compound 12	–	–	0.10

–, no  $R_f$  values determined



**Figure 1:** (a) TLC plates viewed under UV 302nm, (b) TLC plates viewed after staining with vanillin spray reagent

CEF eluent system showed two compounds at  $R_f$  values 0.80 and 0.94 being active against *S. aureus*, but no active compound against *H. pylori*, *S. sonnei* and *S. Typhimurium* was detected on the plate developed in this eluent system.

In the BEA system, it was observed that the degree of inhibition exhibited by these compounds varied from one organism to the other. All the organisms were inhibited on the plates with active compounds at different  $R_f$  values; for *S. aureus* it was 0.20, while for *H. pylori* it was 0.42 [Table 2].

### Column chromatography

Attempt was made to isolate the bioactive compound using column chromatography. The BEA combination was used as the eluent while silica gel packed with benzene was used as the solid phase. A total of 12 fractions were eluted from the compounds [Table 3].

The bioautography analysis showed the presence of active compounds, with  $R_f$  values of 0.12, 0.20, and 0.42 [Table 2] on the chromatogram developed in the BEA eluent system. The compound with  $R_f$  value 0.12 was found to be active against all the test organisms, thus proving to have a broad spectrum activity against Gram-positive and Gram-negative bacteria. Similarly, the compound with  $R_f$  value 0.20 also had activity against the Gram-positive and Gram-negative organisms, but lack anti-*H. pylori* activity. However, the compound with  $R_f$  value 0.42 was found to have selective activity against *H. pylori* alone.

The chromatogram developed in the CEF eluent system, showed compounds with  $R_f$  values 0.80 and 0.94 being active against *S. aureus*, and inactive against *H. pylori*. Activities against *S. sonnei* and *S. Typhimurium* were not reported since the organisms did not grow on the TLC plate; this is probably due to the difficulty of removing residual formic acid left on the chromatogram that inhibited the bacterial growth as previously suggested.<sup>[24]</sup> Various Compounds have been isolated from this plant and they include: friedelin, taraxerone, epifriedelinol, taraxerol, gallic acid, ellagic acid, and  $\beta$ -sitosterol.<sup>[1]</sup> It is likely that the activity seen on the bioautography assay may be elicited by similar compounds. Hence, in a bid to isolate these compounds, column chromatography was performed of which a total of twelve fractions were eluted. Of these, three fractions were found to be active after comparing their  $MIC_{50}$  values with the other fractions. However, it was observed that the  $MIC_{50}$  values of all the eluted fractions against *H. pylori* were found to be higher than that of the other organisms, thus making it the most resistant of all the organisms in this study.

### $MIC_{50}$ determination of eluted fractions

$MIC_{50}$  was carried out on all the fractions eluted from column chromatography in order to assay their antimicrobial

**Table 2:  $R_f$  values of bioactive components of Benzene/Ethanol/Ammonia and Chloroform/Ethyl Acetate/Formic acid chromatograms**

Organism	BEA		CEF	
	a	b	a	b
<i>S. aureus</i>	0.12	+	0.80	+++
	0.20	++	0.94	+++
<i>S. sonnei</i>	0.12	+		*
	0.20	++	×	*
<i>S. typhimurium</i>	0.12	+		*
	0.20	++	×	*
<i>H. pylori</i> 252c	0.12	++		*
	0.42	+	—	*

a:  $R_f$  values of compounds, b: Relative degree of inhibition, ×: No growth, —: No activity, +: Slight inhibition, ++: high inhibition, +++: Very high inhibition, \*: No inhibition recorded

**Table 3: Eluted fractions and their masses**

Compound fractions	Mass (g)
Fraction 1	0.1
Fraction 2	3.32
Fraction 3	0.17
Fraction 4	0.08
Fraction 5	0.06
Fraction 6	0.04
Fraction 7	0.02
Fraction 8	0.03
Fraction 9	0.01
Fraction 10	0.01
Fraction 11	0.01
Fraction 12	0.02

**Table 4:  $MIC_{50}$  values of eluted fractions and ciprofloxacin (mg/mL)**

Test organism	BEA 3	BEA 7	BEA 8	Ciprofloxacin
<i>S. aureus</i>	0.0048	0.0024	0.0024	0.000097
<i>S. sonnei</i>	0.039	0.312	0.078	0.000097
<i>S. Typhimurium</i>	0.312	0.156	0.312	0.0000122
<i>H. pylori</i> 252c	1.25	5	2.5	0.0078

activity. Three fractions labelled BEA3 (fraction 3), BEA7 (fraction 7), and BEA8 (fraction 8) were active. The  $MIC_{50}$  of these active fractions were then compared with that of the control antibiotic [Table 4].

The  $MIC_{50}$  of BEA3 ranged from 0.0048 to 1.25mg/mL, BEA7 0.0024 to 5mg/mL, and BEA8 0.0024 to 2.5mg/mL. The eluted fractions exhibited a stronger activity against all the test organisms as evidenced by their  $MIC_{50}$  value except for *H. pylori* 252c. However there was no statistical significant difference between the  $MIC_{50}$  of the eluted fractions and antibiotic ( $P = 0.546$ ).

## CONCLUSION

The ethyl acetate extract of the stem bark of *B. micrantha* can provide lead molecules which could be useful substrate for the synthesis of new broad spectrum antibiotics for the treatment of infections caused these organisms. Further purification, identification and characterization of the active compounds would be our priority in future studies.

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**Cite this article as:** Adefuye AO, Ndip RN. Phytochemical analysis and antibacterial evaluation of the ethyl acetate extract of the stem bark of *Bridellia micrantha*. *Phcog Mag* 2013;9:45–50.

**Source of Support:** National Research Foundation South Africa (grant reference CSUR2008052900010), and the Govan Mbeki Research and Development Centre, University of Fort Hare, South Africa.

**Conflict of Interest:** No.