# The potential use of leaf extracts of two *Newtonia* (Fabaceae) species to treat diarrhoea

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## Highlights

- *Newtonia hildebrandtii* and *N. buchananii* had strong activity against *E. coli*.
- Extracts had good anti-inflammatory and antioxidant activity, and inhibited acetylcholinesterase.
- *N. buchananii* had excellent antibacterial activity and promising selectivity index of 19.
- These extracts may be developed as potential antidiarrhoeal agents.

## Abstract

In southern Africa, many medicinal plants are used traditionally to treat gastrointestinal disorders and other infectious diseases. *Newtonia hildebrandtii* and *Newtonia buchananii* are used for the treatment of wounds and skin conditions, and for an upset stomach. Following promising unpublished antibacterial activity against *E. coli*, this study was undertaken to evaluate the potential efficacy of *Newtonia hildebrandtii* and *Newtonia buchananii* extracts in treating diarrhoea. The antimicrobial activity of *N. hildebrandtii* and *N. buchananii* extracts was determined against a panel of Gram-positive and Gram-negative bacteria and two fungi implicated in causing diarrhoea. *N. buchananii* had strong antimicrobial effect against *Pseudomonas aeruginosa* with minimum inhibitory concentration (MIC) of 20 µg/ml and moderate activity of 40 µg/ml against *Bacillus cereus*. Cytotoxicity of extracts was evaluated

against Vero cells and both plants were relatively non-toxic with $IC_{50}$ values of 30-750 µg/ml. Selectivity index values as high as 18.75 were reached with the methanol-dichloromethane leaf extract of *N. buchananii*. The extracts also had good anti-inflammatory and antioxidant activities as well as acetylcholinesterase inhibition efficacy. These activities are all useful adjuncts, together with antimicrobial effects, in potential plant-based remedies to be used as diarrhoea treatments.

**Keywords**:

*Newtonia*; Fabaceae; diarrhoea; antimicrobial; cytotoxicity; anti-inflammatory; acetylcholinesterase.

**List of abbreviations**: AchEI, Acetylcholinesterase inhibitors; MIC, minimum inhibitory concentration; DPPH, 2,2' -diphenyl-1-picryhydrazyl; ABTS, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 15-LOX, 15-lipoxygenase; Nb1, *N. buchananii* acetone leaf extract, Nb2, *N. buchananii* methanol-DCM leaf extract; Nb3, *N. buchananii* acetone stem extract; Nb4, *N. buchananii* methanol:DCM seed extract; Nh1, *N. hildebrandtii* acetone leaf extract; Nh2, *N. hildebrandtii* methanol-DCM leaf extract; Nh3, *N. hildebrandtii* methanol:DCM stem extract; NO, nitric oxide; LPS, lipopolysaccharide; MeOH-DCM, methanol-dichloromethane; IL-1β, interleukin-1-beta; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; COX, cyclooxygenase; iNOS, inducible nitricoxide; Ach, acetylcholine; INT, p-iodonitrotetrazolium violet; MEM, Minimal Essential Medium; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; SI, selectivity index; PSF, penicillin-streptomycin-fungizone; DMEM, Dulbecco's Modified Eagle's Medium; $LC_{50}$, lethal concentration that caused 50% of inhibition of cell viability.

## 1. Introduction

Diarrhoea is a major gastrointestinal disorder with multifaceted pathogenesis, including infection by a wide range of microbial pathogens, intestinal inflammation and food allergy as well as drug intolerance (Ahmed, 2012). It has high mortality and morbidity especially in children and immunocompromised patients, and is becoming a neglected disease (Liu *et al*., 2012). The World Child Mortality Report revealed that 5.9 million children under the age of five die every year, and diarrhoea is the cause of 14% of these deaths (WHO, 2015). No widely available source is able to provide accurate estimates of the overall incidence of diarrhoea in developing countries (De Melo *et al*., 2008; Walker *et al*., 2012). Diarrhoea is recognized as the third highest killer in South Africa (CSIR, 2009). Current allopathic medications are inadequate due to resistant pathogens and excessive cost, especially in rural communities of some developing countries, thus the need for alternatives has created a significant challenge.

Following the occurrence of antibiotic resistance, research has been conducted on the screening of a large number of medicinal plants for their potential antimicrobial activity (Nascimento *et al*., 2000; Vlietinck *et al*., 1995). The investigation of traditional medicine as an alternative form to the use of antibiotics as a mechanism of reducing the development of antibiotic resistance has led to exploration of antimicrobial activity of medicinal plants (Dey *et al*., 2014). Plants have been exploited for their medicinal purposes for thousands of years, and have played a significant role in maintaining human health and improving the quality of human life (Samuelsson, 2004; Dzoyem *et al*., 2013). Plants are also regarded as the sleeping giants of drug development and it is hoped that screening plants could offer natural sources of antimicrobial drugs essential for controlling some infections globally (Farnsworth and Morris 1976; Akinpelu and Onakoya, 2006). In southern Africa, medicinal plants are used

traditionally to treat gastrointestinal disorders and other infectious diseases. *Newtonia hildebrandtii* (Vatke) Torre (Figure 1) and *N. buchananii* (Baker) G.C.C.Gilbert & Boutiqu (Figure 2) of the family Fabaceae are medically used for the treatment of wounds and skin conditions, and for an upset stomach (Fratkin, 1996; Kariba and Houghton, 2001). The antimicrobial activity of the stem-bark extracts of *N. hildebrandtii* has been reported (Kariba and Houghton, 2001). However, no work has been recorded on other parts of the plant. To the best of our knowledge, there are no other reports of traditional use, pharmacological activity and phytochemistry of *N. hildebrandtii* and *N. buchananii*.

**Figure 1** ***Newtonia hildebrandtii*** (Photo taken by Professor Jacobus N. Eloff at Lowveld National Botanical Garden in Nelspruit, South Africa)

**Figure 2** ***Newtonia buchananii*** (Photo taken Professor Jacobus N. Eloff at Lowveld National Botanical Garden in Nelspruit, South Africa)

The interrelation of neurology and gastrointestinal systems has been thoroughly studied and established (Perkin and Murray-Lyon, 1998; Nathanson *et al*., 2010; Rao and Gershon, 2016). According to Wood *et al*. (1999), neural networks responsible for controlling digestive functions are positioned in the brain. The severity of diarrhoea may on occasion be due to a neurological disorder. Generally, the digestive tract is muscular and contracts rhythmically to conduct peristalsis. The enteric nervous system (commonly known as the gut's brain) is the intrinsic nervous system of the gastrointestinal tract and its characteristics are related to motility, secretion, digestion, and inflammation (Costa *et al*., 2000; Nezami and Srinivasan, 2010; Sasselli *et al*., 2012). Diarrhoea results following frequent contractions of the GIT and this often reflects a low level of acetylcholinesterase released by the brain.

Acetylcholinesterase inhibitors (AchEI) are the mainstay of pharmacological therapy for neurological diseases such as Alzheimer's disease, dementia, Parkinson's disease and other neurological or psychiatric behavioural dysfunctions. As outlined by Mimica and Presečki

(2009), AchE increases the levels of acetylcholine (Ach) in the brain and diarrhoea has been recognized as the most common gastrointestinal side effect associated with the cholinergic mechanism (Yaari *et al*., 2008).

Diarrhoea causes an imbalance in the gut microbiota, increased gut permeability and inflammation of the intestine (Salminen *et al*., 1996). During inflammation, the intestine is unable to reabsorb as much water from the stool (bowel contents) as it normally would. Inflammation is viewed as a major process involved in the healing of damaged tissues, and results in an increased number of leukocytes and a variety of complex mediator molecules (Damte *et al*., 2011; Oyungerel *et al*., 2013). Among the leukocytes, macrophages are identified as key players in the modulation of the immune inflammatory system (Calandra and Roger, 2003; Fujiwara and Kobayashi, 2005). Macrophages are major sources of pro-inflammatory cytokines and enzymes, such as interleukin-1-beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), cyclooxygenase (COX) and inducible nitric oxide (iNOS) (Duffield, 2003; Bonizzi and Karen, 2004; Verma *et al*., 2010). According to Damte *et al*. (2011) the genes of pro-inflammatory mediators are strongly induced during inflammation and are responsible for its initiation and persistence. Anti-inflammatory agents may also have antioxidant and radical-scavenging mechanisms as part of their overall activity. Plants are recognized as a potential source of natural antioxidants and offer hope to reduce the risk of diseases. They contain bioactive components that can potentially strongly inhibit the expression of lipoxygenase and nitric oxide. The hypothesis from this study is that if medicinal plants have potential to inhibit acetylcholinesterase and also inhibit nitric oxide, then not only will the plants be addressing the challenge of diarrhoea but this will also be an advantage to neurological diseases as NO is a known neurotransmitter and anti-inflammatory

component. Moreover, plants with high antimicrobial activity and low toxicity have the potential to be used to combat diarrhoea in humans and animals.

In this study, *in vitro* antimicrobial, cytotoxicity, anti-inflammatory, antioxidant, and acetylcholinesterase (AchE) inhibitory activities of the acetone and MeOH:DCM (1:1) extracts of *Newtonia hildebrandtii* and *Newtonia buchananii* were evaluated.

## 2. Materials and Methods

### *2.1. Plant material and extraction*

The plant species were collected from the Lowveld National Botanical Garden in Nelspruit, Mpumalanga, South Africa in summer months. Voucher specimens (PRU 122347 for *N. hildebrandtii* and PRU 122348 for *N. buchananii*) were prepared and lodged in the H.G.W.J. Schweickerdt Herbarium at the University of Pretoria (South Africa) for reference purposes. The collected plant materials were dried at room temperature in a well-ventilated room and ground to a fine powder in a Macsalab Mill (Model 2000 LAB Eriez). One gram of each plant part (leaves and stems, and in the case of *N. buchananii* also the seeds and seedpods combined) was separately extracted in 10 ml of pure acetone and 1:1 MeOH-DCM (technical grade, Merck) in polyester centrifuge tubes. The tubes were vigorously shaken for 30 minutes on an orbital shaker, then centrifuged at 4000 x g for 10 min and the supernatant was filtered using Whatman No.1 filter paper before it was transferred into pre-weighed glass honey jar containers. This was repeated thrice on the same plant material and the solvent was removed by evaporation under a stream of air in a fume hood at room temperature to yield the dried extract.

$$\text{Plant crude extraction yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of plant powder extracted}} \times 100$$

### *2.2. Antimicrobial assay*

The antimicrobial potential of the acetone and methanol:DCM extracts dissolved in acetone was determined against the following bacteria: *Staphylococcus aureus* (ATCC 29213), *Bacillus cereus* (ATCC 21366), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 39183) and fungi: *Candida albicans* (ATCC 10231) and a clinical isolate of *Cryptococcus neoformans*. This antimicrobial activity was evaluated in terms of minimum inhibitory concentration (MIC) using a rapid broth microdilution technique with p-iodonitrotetrazolium violet (INT) as a growth indicator as developed by Eloff (1998a). The amount of seeds and seedpods available for harvesting was unfortunately not enough to produce sufficient extracts for use in all the assays so there were only tested for antimicrobial activity. The negative controls were the solvents used to resuspend the extracts, as well as the broth that was used to inoculate a colony for an overnight culture.

### *2.3. Cytotoxicity assay*

The cytotoxicity of the extracts against African green monkey (Vero) kidney cells was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay as previously described by Mosmann (1983) with slight modifications. Vero cells were maintained at 37°C and 5% $CO_2$ in a humidified environment in Minimal Essential Medium (MEM) containing L-glutamine (Lonza, Belgium) and supplemented with 5% fetal bovine serum (Capricorn Scientific Gmbh, South America) and 1% gentamicin (Virbac, RSA). These cells were seeded at a density of $10^5$ cells/ml (100 µl) in 96-well microtitre plates and incubated at 37°C overnight to allow attachment. After incubation, extracts (100 µl) at varying final concentrations were added to the wells containing cells. Doxorubicin hydrochloride (Pfizer) was used as a positive control. A blank containing only the fresh

medium and a negative control made of cells in fresh medium without treatment were used and the plates were further incubated at 37°C and 5% $CO_2$ for 48 hours. After incubation, the medium was aspirated from the cells, which were then washed with 200 µl of phosphate-buffered saline (PBS). Then, 200 µl of the fresh medium with 30 µl of MTT (5 mg/ml in PBS) were added to each well and the plates were incubated at 37°C in a 5% $CO_2$ humidified incubator for 4 hours. The medium was carefully aspirated from the wells and 50 µl of dimethylsulfoxide (DMSO) was added to solubilize the formed formazan crystals and the plates were placed on an orbital shaker for about 2 minutes. The absorbance was measured on a microplate reader (BioTek Synergy) at 570 nm. Cell growth inhibition for each extract was expressed in terms of $LC_{50}$ values, defined as the lethal concentration that caused 50% of inhibition of cell viability. The selectivity index (SI) values were calculated by dividing $LC_{50}$ values by the MIC values ($SI=LC_{50}/MIC$). Tests were carried in quadruplicate and each experiment was repeated thrice.

### *2.4. Anti-inflammatory assays*

The anti-inflammatory activity of the extracts was determined by examining the inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS) activated RAW 264.7 macrophages cells and 15-lipoxygenase enzyme inhibition.

#### *2.4.1. Nitric oxide inhibitory assay*

The RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and were grown in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (4.5 g/L) containing L-glutamine (4 mM) and sodium-pyruvate (HycloneTM) supplemented with 10% (v/v) fetal bovine serum (Capricorn Scientific Gmbh, South America) and 1% penicillin-streptomycin-fungizone (PSF) at 37°C with 5% $CO_2$ in a humidified environment. The RAW 264.7 macrophages were seeded at a density of $2\times10^4$

cells per well in 96 well-microtitre plates and the cells were allowed to attach overnight. The cells were activated by incubation in a medium containing 5 µg/mL of LPS alone (negative control) and treated simultaneously with different concentrations of the samples dissolved in DMSO. After 24 h of incubation at 37°C with 5% $CO_2$ in a humidified environment, the supernatant (100 µl) from each well of the 96 well-microtitre plates was transferred into new microtitre plates and an equal volume of Griess reagent (Sigma Aldrich, Germany) was added. The mixture was left in the dark at room temperature for 15 min and the absorbance was determined at 550 nm on a microplate reader (Synergy Multi-Mode Reader, BioTek). The quantity of nitrite was determined from a sodium nitrite standard curve. The percentage of NO inhibition was calculated based on the ability of each sample to inhibit nitric oxide production by RAW 264.7 macrophages cells compared with the negative control (cells treated with LPS without samples). Subsequently, the cell viability was determined using the MTT assay (Mosmann, 1983) as described previously. Quercetin was used as a positive control for the reduction of NO production (Mu *et al*., 2001).

### *2.4.2. 15-lipoxygenase inhibition assay*

Lipoxygenase inhibition was determined spectrophotometrically based on the formation of the complex Fe3+/xylenol orange as described by Pinto *et al*. (2007). Briefly, 20 µl of Tris-HCl Buffer (pH 7.4) was added to all wells of the 96-well microplates. Then, 20 µl of the extracts (1 mg/ml) were added in the first row of the microplate followed by a two-fold serial dilution. The extract was added to the sample and the sample blank wells. Quercetin served as the positive control, and the buffer was used as a negative control. After the serial dilution, 40 µl of the lipoxygenase enzyme (Sigma Aldrich, Germany) was added to each well and the plates were incubated at room temperature for 5 min. After incubation, 40 µl of linoleic acid (final concentration, 140 µM) prepared in Tris-HCl buffer (50 mM, pH 7.4) was added to the

well (except for the blanks). The plates were incubated at 25°C for 20 min in the dark. Following the incubation was the addition to all wells of 100 µl of freshly prepared ferrous oxidation−xylenol orange (FOX) reagent [sulfuric acid (30 mM), xylenol orange (100 µM), iron (II) sulfate (100 µM) in methanol/water (9:1)]. The plates were incubated at 25°C for 30 min in the dark and 40 µl of linoleic acid was then be added to the blanks. The absorbance was measured at 560 nm using a BioTek Synergy microplate reader.

### *2.5. Antioxidant assays*

The antioxidant activity of the extracts was determined using 2,2' -diphenyl-1-picryhydrazyl (DPPH) and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) assays.

#### *2.5.1. DPPH radical scavenging assay*

The DPPH assay was performed using the method proposed by Brand-Williams *et al*. (1995) and slightly modified by Dzoyem et al. (2014). Ascorbic acid and trolox served as positive controls, methanol as negative control and extract without DPPH as blank. Samples were dissolved in methanol and 40 µl of each sample was added to the 96-well microtitre plates and serially diluted two-fold. Then, a volume of 160 µl of methanolic solution of DPPH was added to each well and the plates were incubated at room temperature in the dark for 30 minutes. The absorbance was then measured at 517 nm using a microplate reader (Epoch, BioTek). The free-radical scavenging activity of each sample and the positive controls were determined as percentage inhibition. The concentration of samples reducing 50% of the free-radical DPPH ($IC_{50}$) was determined by plotting percentage inhibition against the concentrations. The assay was repeated thrice and the results are expressed as mean ± standard deviation.

### *2.5.2. ABTS radical scavenging assay*

The ABTS radical scavenging capacity of the extracts was determined using the method of Re *et al*. (1999) modified by Dzoyem et al. (2014). The ABTS radical stock solution was prepared by reacting 7 mM solution of ABTS and 2.45 mM solution of potassium persulfate at room temperature for 12 hours. The absorbance of ABTS was adjusted to 0.8-0.9 before proceeding with the experiment. Ascorbic acid and trolox served as positive controls, methanol as negative control and extract without ABTS as blank. Samples were dissolved in methanol and 40 µl of the sample was added to the 96-well microtitre plates and serially diluted two-fold. Then, a volume of 160 µl of methanolic solution of ABTS was added to each well and the plates were incubated at room temperature in the dark for 6 min. The absorbance was then measured at 734 nm using a microplate reader (Epoch, BioTek). The free-radical scavenging activity of each sample and the positive controls was determined as percentage inhibition. The $IC_{50}$ values were calculated from the graph plotted as percentage inhibition against the concentrations. The assay was repeated thrice and the results are expressed as mean ± standard deviation.

### ***2.6. Acetylcholinesterase assay***

The acetylcholinesterase (AchE) inhibitory activity was determined spectrophotometrically using Ellman's colorimetric method (Ellman *et al*., 1961) with slight modifications. Twenty-five (25) µl of DMSO were first introduced in all wells of a 96 well-flat bottom microtitre plate. Then, 25 µl of test sample, negative (DMSO) and positive control were added in the appropriate wells of the plate. A volume of 125 µl of AChE (0.2 U/mL) was added to each well and the plates were incubated at room temperature (25°C) for 5 minutes. Next, a volume of 125 µl of Ellman's reagent [3 mM of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) prepared in Buffer A (50 mM Tris–HCl, pH 8.0, containing 0.1 M NaCl and 0.02 M MgCl2·6H2O)]

was added to all wells and this was followed by the addition of 50 µl of Buffer B (50 mM, pH 8, containing 0.1% bovine serum albumin) in all wells. The plates were incubated at room temperature (25°C) for 10 minutes. The absorbance was measured at 405 nm to blank the extract. A 25 µl aliquot of substrate [15 mM of acetylthiocholine iodide (ATCI) in water] was added to all wells and the plates were incubated at room temperature (25°C) for 5 minutes and the absorbance was measured at 405 nm. The assay was performed in triplicate and the $IC_{50}$ values of samples leading to 50 % inhibition were calculated by plotting the percentage of inhibition against the concentrations.

### *2.7. Phytochemical evaluation*

#### *2.7.1. Total phenolic content*

Total phenolic content of the plant extracts was assessed using the Folin-Ciocalteu method (Makkar, 2003) with some modifications. Gallic acid served as the standard to determine total phenolic content. A 50 µl aliquot of freshly prepared 50% methanol (MeOH) plant extracts were transferred into test tubes in quintuplicate. An amount of 950 µl of sterile distilled water was added to the tubes, which were taken to the dark for the addition of 500 µl of 1N Folin-C reagent and 2.5 ml of 2% sodium carbonate. Likewise, a blank containing 50% MeOH instead of the plant extract was prepared. All tubes were incubated at room temperature in the dark for 40 min and aliquots of 200 µl were transferred to microtitre plates to measure the absorbance at 725 nm using a microplate reader (Epoch, BioTek).

#### *2.7.2. Total tannin content*

Total tannin content of the plant extracts was determined using the polyvinylpyrrolidone (PVPP) binding method as described by Makkar (2003). A bound mixture was prepared by mixing 100 mg of PVPP, 1 ml distilled water and 1 ml plant extract in a 50 ml centrifuge

tube. The tube was centrifuged at 1000 rpm for 10 minutes and the thoroughly mixed blend was kept for 15 min at 4°C, and filtered. The filtrate was transferred into a test tube and the total phenolic content was evaluated as described in section 1. Non-tannin phenolic constituents were determined using the standard curve equation y= 4.9022x + c. where y is absorbance, x is gallic acid in mg, and C is 0. The tannin content was calculated as the difference between the total phenolic and non-phenolic content of the plant extracts because the theory is that the tannin would be bound and precipitated by PVPP.

### *2.7.3. Flavonol content*

The flavonol content of the plant extracts was evaluated by the aluminium chloride method (Abdel-Hamed *et al*., 2009) with some modifications. In quintuplicate, 1 ml of each plant extract was mixed with 1 ml of 20 mg/ml of $AlCl_3$ and 3 ml of 50 mg/ml sodium ethanoate in a test tube. The tubes were incubated for 2.5 h and aliquots of 200 µl were transferred to the microtitre plates and to measure the absorbance at 440 nm with microplate reader (Epoch, BioTek). A standard curve was prepared using quercetin in methanol. The amount of flavonol was expressed as mg quercetin equivalent/g of dry plant material.

### *2.7.4. Total flavonoids*

Total flavonoid content of the plant extracts was determined by the aluminium chloride method (Abdel-Hamed *et al*., 2009) with some modifications. In quintuplicate, 100 µl of plant extract was mixed with 100 µl 20% $AlCl_3$ and two drops of glacial acetic acid. The mixture was then diluted with MeOH to 3 000 µl. Blank samples were prepared with the plant extracts without $AlCl_3$ and a standard curve was prepared using quercetin (3.9-500 µg/ml) in methanol. Aliquots of 200 µl were transferred to the microtitre plates and the absorbance was read with a microplate reader (Epoch, BioTek) at 415 nm after 40min

incubation at room temperature. The total flavonoid content was expressed as mg quercetin equivalent/g of dry plant material.

### *2.7.5. Procyanidin content*

Procyanidin content of the extracts was determined using the butanol-HCl assay as described by Makkar (2003). In quintuplicate, 500 µl of plant extract was allotted into the test tubes and diluted to 10 ml with 50% MeOH. A 3 ml of butanol-HCl (95/5%) reagent and 100 µl of 2% ferric ammonium sulphate in 2N HCl were then added to the tube. The tubes were loosely covered and heated in a boiling water bath. The tubes were allowed to cool at room temperature, and aliquots of 200 µl were transferred to microtitre plates to measure the absorbance at 550 nm using a microplate reader (Epoch, BioTek). Absorbance of the unheated mixture was used as blank.

## ***2.8. Statistical analysis***

All experiments were performed in triplicate (quintuplicate in the case of phytochemical evaluation) and the results are presented as mean ± SD (standard deviation) values. Statistical analysis was conducted with GraphPad InStat 3.0 Software and results were compared using the Student-Newman Keuls and Dunnett's tests. Data were analysed using a one-way analysis of variance to compare phytochemical content within each species. Where there were significant differences, a Duncan's Multiple Range Post Hoc test was used to separate the means. A one-sample t-test was used to compare the means between each phytochemical between species. Results were considered significantly different when $p < 0.05$.

## 3. Results and discussion

### *3.1. Antimicrobial activity versus cytotoxic potential against Vero cells*

In this study, acetone and MeOH:DCM extracts were investigate for biological activity and cytotoxicity. Previously, several extraction solvents were assessed for efficacy in extracting plant compounds and it was concluded that acetone was the best extractant (Eloff, 1998b). This solvent extracts a broad spectrum of polar and non-polar components and is miscible with all other solvents. Moreover, acetone exhibits low toxicity to biological organisms in various experiments. The MeOH: DCM mixture can extract compounds of varying polarity (Eloff, 1998b; Mokgotho *et al.*, 2013). Additionally, Eloff (1998b) showed that a 5 minute extraction with finely ground material gave better results than extraction for 24 hours with material that was not so finely ground.

The serial microdilution method was used to determine minimum inhibitory concentrations (MIC) against three Gram-positive bacteria (*Staphylococcus aureus, Bacillus cereus* and *Enterococcus faecalis*), three Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhimurium*) and two fungi (*Candida albicans* and *Cryptococcus neoformans)*. Cytotoxicity against Vero cells using the MTT assay was determined to evaluate the safety of *Newtonia* species under study. The results (Tables 1 and 2) showed that *Pseudomonas aeruginosa* was highly susceptible to the acetone and methanol:DCM (1:1) leaf extracts of *Newtonia hildebrandtii*, and leaf and stem extracts of *N. buchananii*. *N. buchananii* had a strong antimicrobial effect against *P. aeruginosa* with MIC = 20 µg/ml and moderate activity of 40 µg/ml against *B. cereus* while the highest antibacterial activity of *N. hildebrandtii* was against *Bacillus cereus* with MIC = 80 µg/ml. The plant species tested exhibited poor antifungal activity against *Candida albicans* and *Cryptococcus neoformans*. The extracts were relatively non-toxic with $LC_{50}$ values of 30-750 µg/ml. The extracting

**Table 1: Antimicrobial activity and cytotoxicity of the selected *Newtonia* species**

| Plant name | Plant part | Extraction yield (%) | Extract | MIC in mg/ml and test organisms | | | | | | | | $LC_{50}$ (mg/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | **Ec** | **Bc** | **Ef** | **Pa** | **St** | **Sa** | **Ca** | **Cn** | |
| ***N. hildebrandtii*** | Leaves | 23.0 | Acetone | 2.5 | 0.31 | 2.5 | 0.16 | 0.16 | 0.31 | 0.63 | 0.63 | 0.17 |
| | | 28.0 | MeOH/DCM | 2.5 | 0.31 | 2.5 | 0.31 | 0.16 | 0.63 | 0.63 | 0.63 | 0.03 |
| | Stems | 16.0 | Acetone | 2.5 | **0.08** | 2.5 | 0.16 | 0.16 | 0.31 | 0.63 | 0.63 | 0.07 |
| | | 22.67 | MeOH/DCM | 0.63 | 0.16 | 0.31 | 0.63 | 0.16 | 0.63 | 0.63 | 0.63 | 0.15 |
| ***N. buchananii*** | Leaves | 5.67 | Acetone | 0.16 | **0.04** | 0.16 | **0.02** | **0.08** | **0.08** | 0.16 | 0.16 | 0.17 |
| | | 11.00 | MeOH/DCM | 0.31 | **0.08** | 0.16 | **0.04** | **0.08** | 0.31 | 0.63 | 0.63 | 0.75 |
| | Stems | 8.67 | Acetone | 0.31 | **0.08** | 0.31 | **0.02** | **0.08** | 0.31 | 0.63 | 0.63 | 0.12 |
| | | 13.33 | MeOH/DCM | 0.63 | 0.31 | 1.25 | **0.02** | **0.08** | 0.63 | 0.63 | 0.63 | 0.09 |
| | Seeds and | 4.0 | Acetone | 0.63 | 0.16 | 0.63 | 0.16 | 2.5 | 0.31 | 0.31 | 0.31 | 0.03 |
| | seedpods | 5.33 | MeOH/DCM | 1.25 | 0.31 | 0.31 | 0.16 | 1.25 | 0.31 | 0.63 | 0.63 | 0.04 |
| **Gentamicin** | | | | $1.95 \times 10^{-4}$ | $7.8 \times 10^{-4}$ | $1.95 \times 10^{-4}$ | $7.8 \times 10^{-4}$ | $7.8 \times 10^{-4}$ | $7.8 \times 10^{-4}$ | - | - | |
| **Amphotericin B** | | | - | - | - | - | - | - | - | 0.03 | 0.03 | |

Minimum Inhibitory Concentration (MIC); Methanol-Dichloromethane (MeOH/DCM); concentration of a given agent lethal to 50% of the cells ($LC_{50}$). *Escherichia coli* (Ec), *Bacillus cereus* (Bc), *Enterococcus faecalis* (Ef), *Pseudomonas aeruginosa* (Pa), *Salmonella typhimurium* (St), *Staphylococcus aureus* (Sa), *Candida albicans* (Ca) and *Cryptococcus neoformans* (Cn). Values in bold are considered very active (MIC < 0.1 mg/ml)

**Table 2: Selective index of the selected *Newtonia* species**

| Plant name | Plant part | Extract | Test organisms and selectivity index (SI) = $LC_{50}$/MIC | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **Ec** | **Bc** | **Ef** | **Pa** | **St** | **Sa** | **Ca** | **Cn** |
| ***N. hildebrandtii*** | Leaves | Acetone | 0.07 | 0.55 | 0.07 | **1.06** | **1.06** | 0.31 | 0.27 | 0.27 |
| | | MeOH/DCM | 0.01 | 0.10 | 0.01 | 0.10 | 0.19 | 0.05 | 0.05 | 0.05 |
| | Stems | Acetone | 0.03 | 0.92 | 0.03 | 0.46 | 0.46 | 0.24 | 0.11 | 0.11 |
| | | MeOH/DCM | 0.24 | 0.94 | 0.48 | 0.24 | 0.94 | 0.24 | 0.24 | 0.24 |
| ***N. buchananii*** | Leaves | Acetone | **1.07** | **4.29** | **1.07** | **8.57** | **2.14** | **2.14** | **1.06** | **1.06** |
| | | MeOH/DCM | **2.42** | **9.38** | **4.69** | **18.75** | **9.38** | **2.42** | **1.19** | **1.19** |
| | Stems | Acetone | 0.39 | **1.49** | 0.39 | **5.96** | **1.49** | 0.39 | 0.19 | 0.19 |
| | | MeOH/DCM | 0.14 | 0.28 | 0.07 | **4.35** | **1.09** | 0.14 | 0.14 | 0.14 |
| | Seeds and | Acetone | 0.05 | 0.18 | 0.05 | 0.18 | 0.01 | 0.09 | 0.10 | 0.10 |
| | seedpods | MeOH/DCM | 0.03 | 0.14 | 0.14 | 0.26 | 0.03 | 0.14 | 0.06 | 0.06 |

Selectivity Index (SI); Methanol-Dichloromethane (MeOH/DCM). *Escherichia coli* (Ec), *Bacillus cereus* (Bc), *Enterococcus faecalis* (Ef), *Pseudomonas aeruginosa* (Pa), *Salmonella typhimurium* (St), *Staphylococcus aureus* (Sa), *Candida albicans* (Ca) and *Cryptococcus neoformans* (Cn). Values in bold highlight promising SI values.

solvents (negative controls) had no effect on the organisms at the concentrations used. Selectivity index values as high as 18.75 were reached with *N. buchananii* methanol:DCM (1:1) leaf extract. The acetone extracts of both *N. buchananii* and *N. hildebrandtii* had good antimicrobial activity against a range of bacterial strains implicated in diarrhoea. The high antibacterial activity against *Pseudomonas aeruginosa* and the high cellular safety (low cytotoxicity) proposes potential for development from these extracts of safe, effective agents against Gram-negative bacteria.

### *3.2. Nitric oxide (NO) inhibitory activity of extracts on LPS-activated RAW 264.7 macrophages*

Figure 1 describes the effects of *N. hildebrandt*i and *N. buchananii* extracts on the percentage of nitric oxide inhibition, nitric oxide production and cell viability on LPS-activated RAW 264.7 macrophages cells. It is observed from this figure that all extracts excluding Nb4 (*N. buchananii* methanol:DCM seed extract) were not toxic at the concentrations tested. Plant extracts with good nitric oxide inhibitory activity and low cytotoxicity are beneficial. A concentration-dependent inhibition of the extracts was observed, and the extracts Nh1 (*N. hildebrandtii* acetone leaf extract), Nh2 (*N. hildebrandtii* methanol-DCM leaf extract) and Nh3 (*N. hildebrandtii* methanol:DCM stem extract) revealed the strongest anti-inflammatory potential (Table 3). The *N. hildebrandtii* methanol:DCM leaf extract (Nh2) had the highest NO inhibitory activity with $IC_{50}$ of 76.82 µg/ml. Nitric oxide plays a significant role in the inflammatory process and is synthesized by inducible nitric oxide synthase (iNOS) with response to lipopolysaccharide (LPS) (Oyungerel *et al*., 2013). However, the overproduction of nitric oxide may lead to inflammation and cytotoxicity (Taylor *et al*., 1997; Stoclet *et al*., 1998; Hirai *et al*., 2014). Quercetin has been described for its ability to suppress nitric oxide production in LPS-stimulated RAW 264.7

**Table 3: Anti-inflammatory, antioxidant, and acetylcholinesterase activity of the selected *Newtonia* species**

| Plant name | Plant part | Extract | Nitric oxide ($IC_{50}$ in µg/ml) | Lipoxygenase activity ($IC_{50}$ in µg/ml) | Antioxidant assay ($IC_{50}$ in µg/ml) | | Acetylcholinesterase activity ($IC_{50}$ in µg/ml) |
|---|---|---|---|---|---|---|---|
| | | | | | DPPH | ABTS | |
| ***N. hildebrandtii*** | Leaves | Acetone | 95.97±5.98[a] | 27.39±1.41[a] | 0.36±0.08[a] | 1.51±0.00[a] | NA |
| | | MeOH/DCM | 76.82±3.81[b] | 47.82±2.69[b] | 0.29±0.20[a] | 1.19±0.21[a] | NA |
| ***N. hildebrandtii*** | Stems | Acetone | 99.61±10.97[a] | 15.51±0.39[c] | 0.34±0.02[a] | 0.21±0.08[b] | NA |
| | | MeOH/DCM | > 100[a] | 16.58±3.23[c] | 0.72±0.05[b] | 0.63±0.01[c] | NA |
| ***N. buchananii*** | Leaves | Acetone | > 100[a] | 13.85±0.25[c] | 0.63±0.22[b] | 1.04±0.10[a] | NA |
| | | MeOH/DCM | > 100[a] | 14.82±2.99[c] | 0.33±0.06[a] | 0.09±0.02[d] | 220.01±18.73[a] |
| ***N. buchananii*** | Stems | Acetone | > 100[a] | 14.35±2.17[c] | 0.46±0.07[d] | 0.50±0.10[c] | 505.80±0.00[b] |
| | | MeOH/DCM | > 100[a] | 21.46±2.31[d] | 0.49±0.09[d] | 0.01±0.00[e] | NA |
| **Controls** | Ascorbic acid | | | | 0.02±0.00[e] | 0.37±0.36[c] | |
| | Trolox | | | | 0.13±0.00[f] | 0.66±0.05[c] | |
| | Quercetin | | 14.20±1.94[c] | 24.60±0.70[d] | | | |
| | Eserine | | | | | | 1.37±0.01[c] |

Data are presented as average of triplicate measurements ± standard deviation; for each parameter within a column of the above table, values with different letters mean significantly different at $p < 0.05$ while values with same letters are statistically not different. Methanol-Dichloromethane (MeOH/DCM); NA: extract not active at the tested concentration.

murine macrophage cell line (Mu *et al*., 2001). There was significant production of nitric oxide by Nh1 (*N. hildebrandtii* acetone leaf extract), Nh2 (*N. hildebrandtii* methanol-DCM leaf extract) and Nh3 (*N. hildebrandtii* methanol-DCM stem extract). The inhibition of NO production by *N. hildebrandtii* observed in the RAW 264.7 macrophages indicated that antioxidant molecules may be associated with the inhibitory activity.

### *3.3. Lipoxygenase (LOX) inhibitory activity of extracts*

Figure 2 represents the potential of the extracts to inhibit lipoxygenase activity of extracts. Results show that Nb1 (*N. buchananii* acetone leaf extract), Nb2 (*N. buchananii* methanol-DCM leaf extract) and Nb3 (*N. buchananii* acetone stem extract) had the highest lipoxygenase inhibitory potential with $IC_{50}$ values of 13.85, 14.82 and 14.35 µg/ml respectively. The anti-inflammatory effect of *N. hildebrandtii* and *N. buchananii* indicates that the plant species contain bioactive components that can strongly inhibit the expression of NO and LOX. The identification of anti-inflammatory compounds from these *Newtonia* species may be significant in future development of remedies for diarrhoea and related conditions.

### *3.4. Antioxidant effect of the extracts*

Anti-inflammatory agents may also have antioxidant and radical-scavenging mechanisms as part of their overall activity. The DPPH assay is dose-dependent and the results showed that *N. hildebrandtii* methanol:DCM leaf extract (Nh2) was most active with $IC_{50} = 0.29$ µg/ml as reported in Figure 3(a). There was also a dose-dependent effect observed in Figure 3(b). *N.*

*buchananii* methanol:DCM leaf extract (Nb2) and Nb4 *N. buchananii* methanol:DCM stem extract (Nb4) were most active in the ABTS scavenging assay (Table 3).

### *3.5. Acetylcholinesterase activity of extracts*

Acetylcholinesterase inhibitory effects were tested and only Nb2 (*N. buchananii* methanol:DCM leaf extract) and Nb3 (*N. buchananii* acetone stem extract) exhibited AChE inhibitory properties with $IC_{50}$ = 220.01 and 505.80 µg/ml, respectively. Diarrhoea results following frequent contractions of the GIT and this often reflects a low level of acetylcholinesterase released by the brain. The ability of *N. hildebrandtii* and *N. buchananii* extracts to inhibit nitric oxide (NO) and acetylcholinesterase respectively encourages the use of the plants in addressing the challenge of diarrhoea. The activity may also be an advantage in neurological diseases as NO is known to be a neurotransmitter as well as an anti-inflammatory component.

### *3.6. Polyphenolic analysis of extracts*

The total phenolic, flavonol, flavonoid and tannin contents were higher in *Newtonia hildebrandtii* leaves than in *N. buchananii* leaves (Table 4). The phytochemical contents were all significantly different ($p > 0.05$) between the two *Newtonia* species except for the flavonol content which was not statistically different. The phytochemicals in these *Newtonia* species may possibly account for the antimicrobial, antioxidant, anti-inflammatory and acetylcholinesterase activities observed in this study. Other scientific reports confirmed that plant extracts with high phenolic and flavonoid content correlate well with the antioxidant and anti-inflammatory activities (Diaz *et al*., 2012; Figueroa *et al*., 2014; Rebaya *et al*.,

**Table 4: Phytochemical analysis of the selected *Newtonia* species**

| Plant name | Plant part | Extract | Total phenolics (mg GAE/g DW) | Total tannins (mg GAE/g DW) | Total flavonoids (mg QE/g DW) | Flavonol content (mg GAE/g DW) | Proanthocyanidin content (mg GAE/g DW) |
|---|---|---|---|---|---|---|---|
| ***N. hildebrandtii*** | Leaves | MeOH | 139.21±6.91[a] | 1.33±0.16[a] | 11.78±0.34[a] | 13.28±2.76[a] | 0.44±0.01[a] |
| ***N. buchananii*** | Leaves | MeOH | 46.89±2.76[b] | 0.48±0.05[b] | 8.35±0.23[b] | 8.33±5.31[b] | 0.50±0.02[a] |

Data are presented as average of quintuplicate measurements ± standard deviation; for each phytochemical tested, values with different letters mean significantly different at $p < 0.05$ while values with same letters are statistically not different. Methanol (MeOH); dry weight (DW); gallic acid equivalent (GAE); quercetin equivalent (QE)

2015). Additionally, tannins are viewed as being strong antioxidants with anti-inflammatory, anti-diarrhoeal, cytotoxic, antiparasitic, antibacterial, antifungal, and antiviral activities (Wink, 2015). The procyanidin content was slightly higher in *N. buchananii* than in *N. hildebrandtii* which may explain the acetylcholinesterase inhibitory activity of this plant. According to Xu *et al*. (2009), procyanidins exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, and anticarcinogen properties. Furthermore, it has been reported that procyanidins cause beneficial changes in the central cholinergic system.

## 4. Conclusion

*Newtonia hildebrandtii* and *N. buchananii* have the potential to scavenge free radicals and modulate inflammatory reactions. The isolation of anti-inflammatory as well as antibacterial compounds from these *Newtonia* species may therefore be beneficial in the pathophysiology of diarrhoea and related conditions. The high antibacterial activity and high cellular safety (low cytotoxicity) as well as acetylcholinesterase inhibitory properties of the *N. buchananii* extracts indicates potential for drug development as safe and effective agents for infectious disease treatment. Of particular interest is the promising activity shown by the *N. buchananii* leaf and stem extracts against *Pseudomonas aeruginosa*, against which resistance to standard antimicrobial therapy is developing at an alarming rate. The mechanism of action of this extract and the compounds responsible for activity are deserving of further attention.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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