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Phytochemical content, antibacterial, antioxidant and cytotoxic activities, of leaves extracts of *Eucalyptus globulus, Peltophorum africanum* and *Vangueria infausta*

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Abstract
This study aimed to determine the extraction yield, phytochemical content, antibacterial, antioxidant, and cytotoxic activities of leaves extracts from *Eucalyptus globulus, Peltophorum africanum,* and *Vangueria infausta*. Leaves were harvested, separated from the stems, and dried for chemical analysis. Crude and oil extraction, antioxidant activity, cytotoxicity, and minimum inhibitory concentration were determined, and tannins, flavonoids and alkaloids were quantified by standard protocols. The phenolic, flavonoids, and condensed tannin contents were higher ($P < 0.05$) in *V. infausta* extract than in *E. globulus* and *P. africanum*. The radical scavenging activities were higher ($P < 0.05$) in *V. infausta, E. globulus* than in *P. africanum*. The antibacterial activity was lower ($P < 0.05$) for *P. africanum* and *E. globulus* oil, and was strongly related to the presence of phenolics and flavonoids. The lack of toxicity of plant extracts suggests that extracts can be used as animal feed additives with no risk of toxicity. *Vangueria infausta*, *Eucalyptus globulus* had the highest antioxidant capability and can thus modulate nutrient metabolism in animals.

Keywords Ethno-veterinary · Mean inhibitory concentration · Proximate analysis · Radical scavenging

1 Introduction

The study and exploration of herbal plants with medicinal properties provide a fascinating outcome, with regards to the growing problem of resistance of the currently available commercial antibiotics [1–3]. The bioprospecting of plants with biological activities has been ongoing and the results from such processes have registered significant improvements in the livestock industry [4–10]. The basis for these activities is predicated on the chemical and phytochemical composition of the plants. In sub-Saharan Africa (SSA) grazing is significantly reduced, forcing animals to browse trees and shrubs to meet their nutritional needs [11–13], however, these plants contain secondary metabolites that provide them the ability to survive harsh environmental conditions [14] as well as deter herbivory. The lack of quality feeds and food has resulted in compromised growth rates [15, 16] and increased prevalence of disease-causing organisms [17]. To counter feed shortages and poor performance by animals, farmers have used antibiotic feed additives in promoting

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efficiency [18–20]. These chemical-based feed additives are expensive and have health implications for the consumer. Again because farmers want to meet their production targets, the persistent use of these antibiotics has resulted in the spread of antibiotic-resistant bacteria to both animals and humans [21]. To this end there is therefore a need to replace antibiotics with naturally-occurring feed additives such as herbal extracts. In agreement Formato et al. [22] suggested the formulation of animal feeds/supplements based on naturally occurring bioactive components as prudent and to be widely acceptable. In recent years, studies to evaluate phytochemical components in plants and trees has gained momentum [9, 23–26] and registered significant effects on animal growth and welfare.

Vangueria infausta, Eucalyptus globulus and *Peltophorum africanum* are among the most browsed plants in Southern Africa. *Eucalyptus globulus* belongs to the *Myrtaceae* family, and is found in various parts of the world [27]. The main active ingredient in *Eucalyptus* oil (eucalyptol, 8-cineole) [28, 29] retain toxicity against a wide range of microorganisms [30], modify rumen metabolism [31, 32], and inhibit the rate of deamination of amino acids [33]. *Vangueria infausta* belongs to the *Rubiaceae* family, seldom browsed by cattle, but very much so by goats, and wild animals such as elephant, giraffe, kudu and nyala. The species possess a number of beneficial volatile compounds [34–37]. *Peltophorum africanum* (weeping wattle) belongs to the *Fabaceae* family, and is inherent to Southern Africa, is well known for its antimicrobial activity [30], antioxidant properties and non-toxicity at low concentrations [38]. Little information is available on the uses of *Vangueria infausta* and *Peltophorum africanum* in animal production or nutrition, nonetheless these species are browsed by ruminants and are a good source of phytochemicals. In addition, the identification and extraction of the active compounds in these species is a missing link in livestock science.

Although polyphenolic compounds are beneficial [39, 40], their widespread use as additives in animal feeding necessitates further research particularly regarding their cytotoxicity. It is assumed, with time, that the identification of active compounds with medicinal properties will lead to drug development to ease on drug resistance by microorganisms. Therefore, the study aimed to determine the chemical and phytochemical composition, quantify the antioxidant, antibacterial and cytotoxicity effects of extracts from leaves of *E. globulus*, *P. africanum* and *V. infausta*.

2 Materials and methods

The experimental protocol and procedures were approved by the Animal Ethics Committee (MUP141SMOS01) of the University of Fort Hare (UFH), Eastern Cape, South Africa. *V. infausta*, *P. africanum* and *E. globulus* were collected in March in Ga-Masemola, 24.5551°S, 29.6345°E, South Africa. The plant material was identified by botanists at Department of Botany UFH, and voucher specimens were deposited at the UFH herbarium with IDs P001, P003 and P004, respectively.

2.1 Chemical characterization of plant leaves

The dry matter (DM) of plant leaves was determined by oven drying at 60 °C for 48 h. Dried samples (six for each plant) were ground and analysed for crude protein (CP) according to AOAC [41] procedure 968.06. The Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analysed based on the method of Van Soest et al. [42] by refluxing ~ 0.45 g of each sample with neutral or acid detergent solution, respectively for one hour using ANKOM 2000 Automated fibre analyser (ANKOM Technology, Fairport, NY). Heat stable bacterial amylase and sodium sulphate were used for NDF determination. The fraction of fibre was expressed as % DM including of residual ash.

2.2 Extraction and determination of extract's yield

2.2.1 Crude extract

Each sample (60 g) was soaked in 450 mL of pure deionized water overnight on a magnetic stirrer (Gerhardt Bonn Shaker). The soaked plant material was thereafter filtered through a filter paper (MN 715, 150 mm). The residue was extracted and the solvent separated from the mixture by reduced pressure at 70 °C using a rotary evaporator. The crude extract was stored in glass bottle at 4 °C until further use. The extraction yield was expressed as:

$$\text{Extraction yield \%} = \frac{\text{Weight of the dry extract (g)}}{\text{Weight of the sample used for extraction (g)}} \times 100.$$

2.3 Essential oils

The extraction of essential oils (EO) was carried out in the laboratory at the Department of Plant Science, Faculty of Agricultural and Biological Science, Pretoria, South Africa. Briefly, 200 g of chopped *E. globulus* leaves, was homogenized in distilled water (250 mL) using a blender for 1 min at medium speed, before being homogenized and macerated for 1 h. The EO was extracted using a vertical steam distillation unit (Vineland, NJ) consisting of a hot plate, boiling flask, biomass flask, still head, condenser and receiver. A 2 L flask with the homogenate was heated for three hours at 100 °C. The condensed vapour was separated through an oil/ water separator. To obtain a final EO yield (ratio final volume of extract/weight of fresh plant) the volume extracted was adjusted to 100 mL using distilled water to obtain the crude EO. The percentage yields were then calculated. All extracts were placed in a bottle and kept at 3–4 °C until further use.

2.4 Quantification of secondary metabolites

2.4.1 Total phenolics

The determination of the total phenolics content of the extracts was done based on Wolfe et al. [43]. Extract (1 mL) was added to 5 mL of Folin-Ciocalteu reagent (10% v/v) and 4 mL of Na_2CO_3 (7.5% w/v). The samples of extract were kept at 1 mg/mL. The mixture was shaken using a vortex and left for 30 min at 40 °C for colour development. The absorbance was measured at 517 nm using a spectrophotometer (HP, New Jersey, and USA). The total phenolic content was expressed as gallic acid equivalents (mg GAE/g). The phenol content was extrapolated from the gallic acid standard/calibration graph equation; $y = 8.766\,8x + 0.1977$, $R^2 = 0.998\,3$, and calculated as:

$$C = c \times V/m,$$

where C = total content of phenolic compounds in mg/g plant extract in gallic acid equivalent (GAE) extract, c = the concentration of gallic acid determined from the calibration curve in mg/ml, V = the volume of extract in mL and m = the weight (g) of extract used.

2.4.2 Flavonoids

The total flavonoid content was analysed according to the method of Ordon et al. [44]. A volume of 0.5 mL of $AlCl_3$ ethanol (CH_3CH_2OH) solution (2% w/v) was mixed with an equal amount of sample solution (2 mg/mL). Extracts were evaluated in triplicate at a final concentration of 1 mg/mL. The resultant mixture was incubated for 1 h until a yellow colour appeared (presence of flavonoid). The absorbance was then measured at 420 nm using Helios Epsilon Thermo Spectronic (USA). Flavonoid content was calculated using the equation $y = 1.1734x + 0.1543$, $R^2 = 0.969\,8$ and the results were expressed as mg of quercetin equivalent (QE)/g using the formula:

$$C = c \times V/m$$

where C = total flavonoids expressed as mg/g plant extract in gallic acid equivalent.

mg QE/g extract, c = the concentration of gallic acid established from the calibration curve in mg/mL, V = the volume of extract in ml and m = the weight (g) of extract used.

2.4.3 Condensed tannins

The same aqueous acetone (7:3 v/v, acetone: water) leaves extract (0.5 mL) was used to assay for soluble condensed tannins (SCT) by the modified method of Porter et al. [45] The extract was pipetted into 3 mL test tube and before adding butanol-($C_4H_{10}O$) HCl reagent. The Folin-Ciocalteu assay was standardized using gallic acid and the butanol-HCl assay with purified-condensed tannins. The test-tube was then closed and heated at 100 °C for 1 h after which, the tubes were cooled to room temperature and absorbance measured at 550 nm using a spectrophotometer (T60 UV–Visible

Spectrophotometer, PG Instruments). Soluble condensed tannin proportion was reported as absorbance units (AU) for each 200 mg sample [46, 47]. Condensed tannins (% in dry matter) as leucocyanidin equivalent were calculated by the formula:

$$(\text{A 550 nm x 78.26 x Dilution factor})/\,(\%\ \text{dry matter}).$$

This formula assumes that the effective El %, 1 cm, 550 nm of leucocyanidin is 460.

2.4.4 Saponins

The saponin content was determined using the method of Shiau et al. [48] Plant extracts samples (5 g) were extracted by maceration at 70 °C for 6 h in 50 mL of methanol (CH_3OH) After cooling, extracts were filtered through a Whatman No 1 filter paper, with methanol vacuum-evaporated at 45 °C for 30 min. After the evaporation, dry weights of plant extracts were 0.20, 0.30, and 0.03 g, respectively. The residues were then suspended in 50 mL of distilled water each and extracted three times by the use of 100 mL of ethyl ether $C_2H_5OC_2H_5$). After the removal, the solutions were extracted with 100 mL and 50 mL of n-butanol (C_4H_9OH), respectively. The butanol fractions were dried by evaporation and the resultant products representing the crude saponin content were expressed as mg of saponin per % of dry plant material weight.

2.4.5 Alkaloids

The quantities of alkaloids were determined based on Harborne [49]. Approximately 200 mL acetic acid (CH_3COOH) (10%) was added to plant sample (5 g). The mixture was covered with foil and left for 4 h before concentrating the filtrate on a water bath to 1/4 of its first volume. Concentrated ammonium hydroxide (NH_4OH) was then added in a drop to the extract until the precipitation was completed. The whole solution was then allowed to settle. The precipitates were washed using diluted ammonium hydroxide (NH_4OH) and then filtered. The residue was then dried and weighed, and the alkaloid content was calculated as:

$$\%\ \text{Alkaloids} = \frac{\text{Final weight of sample}}{\text{Initial weight of extract}}.$$

2.4.6 Antioxidant assays

The total antioxidant capacity of the extract was determined using 2-diphenyl-1picrylhydrazyl (DPPH) radical according to Shen et al. [50]. The DPPH assay was performed using the methanol (CH_3OH) extracts. The plant extracts were first tested for the antioxidative potential. A solution of DPPH (0.135 Mm) in methanol (CH_3OH) was prepared and 1.0 mL added to 1.0 mL of extract (25–500 µg/mL) dissolved in methanol. The DPPH in methanol was used as a negative control. The combination was mixed and left at room temperature for 30 min in the dark before measuring the absorbance at 517 nm (Helios Epsilon Thermo Spectronic, USA) using ascorbic acid ($C_6H_8O_6$) as the reference standard. Tests were done in triplicates. The ability of plant extracts to scavenge DPPH radical was calculated as:

$$DPPH\ scavenging\ acitivity\ (\%) = \frac{Abs\ control\ - Abs\ sample}{Abs\ control} \times 100,$$

where: Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample extract or standard.

The EC_{50} (concentration that allows 50% inhibition) was calculated from the plot of serial dilutions against the % inhibition for each extract sample and the ascorbic acid.

2.4.7 Cytotoxicity assay

The cytotoxicity of the acetone extracts against Veronmonkey kidney cells was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay as previously described by Mosmann [51] with slight modifications. Cells were seeded at a density of 1×10^5 cells/mL (100 µL) in 96-well micro-titre plates and incubated at 37 °C and 5% CO_2 in a humidified environment. After overnight incubation, 100 µL each of varying extract

concentrations were added to the wells containing cells. Actinomycin D was used as a positive drug control. A suitable blank control with equivalent concentrations of acetone was also included and the plates were incubated for 48 h in a CO_2 incubator. Thereafter, the medium in each well was aspirated from the cells, cells were washed with PBS, and finally, 200 µL fresh media was added to each well. Thirty microlitres of MTT (5 mg/mL in PBS) was added to each well and the plates were incubated at 37 °C for 4 h. The medium was aspirated from the wells and DMSO was added to solubilise the formazan crystals. The absorbance was measured using a BioTek Synergy microplate reader at 570 nm. The percentage of cell growth inhibition was calculated based on a comparison with untreated cells. The selectivity index values were calculated by dividing cytotoxicity LC_{50} values by the MIC values of the test bacteria in the same units ($SI = LC_{50}/MIC$).

2.5 Isolation of ruminal bacterial from dairy cow

The isolation and purification was performed using the sterile anaerobic technique as described by Hungate [52] and modified by Byrant [53]. Rumen content was obtained 2 h after the morning feeding from a cannulated, lactating Holstein cow, which had been fed total mixed ration containing 18% crude protein (CP) and 12 MJ kg DM^{-1} energy from Agricultural Research Council (ARC). The rumen content was blended for 10 s and thereafter squeezed through four layers of cheesecloth before being serially diluted in five-fold amounts in saline solution. Pure cultures of microorganisms were inoculated into Wilkins Chalgren Agar (WCA) and was incubated for 24 h at 39 °C. Material from well-isolated colonies was picked from the plates with a bent platinum-iridium wire and stab-inoculated into set WCA. Colonies were selected from plates randomly and as many colonies as possible were picked from the same plates. Inoculated slant cultures was incubated at 39 °C and growth was obtain within two days. Gram stunning was then performed for identification by examining the slide for bacterial organisms using a 100X objective microscope: Gram-positive bacteria, stained deep violet to blue and gram-negative bacteria stained pink to red. The gapped BLAST program was used to identify isolated bacteria strains [54]. The program is based on searching protein and DNA data bases for sequence similarities and automatically combining statistically significant alignments produced by BLAST into a position-specific score matrix and then searching the database using this matrix.

2.6 Minimum inhibitory concentration of crude plant extracts, essential oils, and their antibacterial effects

The minimum inhibitory concentration (MIC) was determined in 96-well microliter plates using the method of Eloff [55] Serial dilutions of the EO was made with Mueller–Hinton broth (0.01% Tween 80) to get at the end of test a concentration which ranged from 0.08 to 12 mg/mL and a final concentration of dimethylsulfoxide (DMSO) of 2.5%. A standardized suspension of isolated bacteria (McFarland No. 1) was made in Mueller–Hinton broth, from which 100 µL of the final inoculum that contained around 1.5×10^6 colony forming units (CFU) was added to the appropriate wells to reach a final volume of 200 µL. Inoculated plates were incubated at 37 °C for 24 h. An hour before the ending the incubation, 40 µL of a 0.2% solution of Iodo-Nitro Tetrazolium (INT) (Merck, South Africa) was added to the wells. The inhibition of growth was detected when the solution in the well remained clear after incubation with INT. The assay was run three times. The lowest concentration of extract that showed no visible growth were recorded as the MIC. Actinomycin D and the bacteria with DMSO were used as the positive the negative control, respectively.

2.7 Statistical analysis

The extract yields and phytochemical measurements were subjected to the analysis of variance (ANOVA) in a completely randomised design where plant extracts were regarded as treatments using SAS [56]. The distribution of measured secondary metabolites was determined using SAS [56]. Linear regressions were performed to establish relationships between EC_{50} values for DPPH and all measured secondary metabolites using SAS [56]. The data from the cytotoxic assay were analysed with Graph Pad Prism 4 software [57] and data on diameter of inhibition (MIC) were subjected to ANOVA using PROC GLM SAS [56]. The statistical model was;

$$Yci = \mu + Ti + \delta c + eci,$$

where Yci = observation value for diameter of inhibition and MIC µ = Overall mean of the population, Ti = fixed effect of the extract plant (*V. infausta, P. africanum* and *E. globulus*), δc = random effect of extract sample, and eci = error.

3 Results

3.1 Chemical composition

The chemical composition of the three plant leaves is presented in Table 1. While *E. globulus* showed lower DM values, its NDF was significantly higher ($P < 0.05$).

3.2 Extract yield

The results of extract (crude extract and oil) yield are presented in Table 2. The period of extraction for essential oil of each extracts was 6 h, with the first hour returning a better yield and from the third hour onwards the volume of essential oil extracted was insignificant. High ($P < 0.05$) yields of both crude extract and oil were observed for *E. globulus*, followed by *P. africanum* and lastly *V. infausta*.

3.3 Secondary metabolites content

The concentrations of the total phenolics, flavonoids, condensed tannin, saponins and alkaloids present in crude extracts of *E. globulus, P. africanum* and *V. infausta* are presented in Table 3. The secondary metabolites were different among ($P < 0.05$) plants extracts; *V. infausta* had the highest ($P < 0.05$) contents of phenolics, flavonoids and condensed tannins, followed by *E. globulus* and lastly *P. africanum*. Saponins and alkaloids were higher ($P < 0.05$) in *E. globulus* than the two other plant extracts, but did not ($P > 0.05$) differ between *V. infausta* and *P. africanum*. In terms

Table 1 Chemical composition of plant leaves of *Eucalyptus globulus, Peltophorum africanum* and *Vangueria infausta*

Chemical composition	Tree species			SEM	P-values
	E. globulus	*P. africanum*	*V. infausta*		
DM, %	59.07[b]	64.43[a]	61.99[a]	2.56	0.003
CP, %	9.58	10.43	10.46	0.46	0.318
NDF, %	41.17[a]	34.57[b]	34.46[b]	1.70	0.002
ADF,%	26.86	23.15	28.87	1.22	0.512
ADL,%	11.29	9.77	14.03	0.63	0.342

DM dry matter, *CP* crude protein, *NDF* neutral detergent fibre, *ADF* acid detergent fibre, *ADL* acid detergent lignin

[abc]Means are significant at $P < 0.05$.

Table 2 Yield of essential oils from plant leaves of *Eucalyptus globulus, Peltophorum africanum* and *Vangueria infausta*

	E. globulus	*P. africanum*	*V. infausta*	*SEM*	P-value
Leaves weight (g)	200	200	200		
% Crude plant extracts	9.20[a]	1.20[b]	0.97[b]	1.34	0.001
% oil	3.86[a]	0.08[b]	0.03[b]	0.05	0.001

[abc]Means in the same row (% crude extract and % oil) with different superscripts differ ($P < 0.05$)

Table 3 Secondary metabolites in *Eucalyptus globulus, Peltophorum africanum* and *Vangueria infausta*

Parameter	Treatments			SEM	P-value
	E. globulus (CE)	*P. africanum* (CE)	*V. infausta* (CE)		
Phenolics (mg GAE/g)	181.83[b]	50.98[c]	200.66[a]	2.56	< 0.001
Flavonoids (mg QE/g)	175.02[b]	62.93[c]	191.49[a]	1.70	< 0.001
Condensed tannins (mg CE/g)	7.01[b]	0.50[c]	120.39[a]	0.46	< 0.001
Saponins (%)	15.24[a]	9.33[b]	10.50[b]	1.22	0.03
Alkaloids (%)	8.48[a]	2.83[b]	2.54[b]	0.63	0.001

[abc]Means in the same row with different superscripts differ ($P < 0.05$). GAE: gallic acid equivalent, QE: quercetin equivalent, CE: catechin equivalents

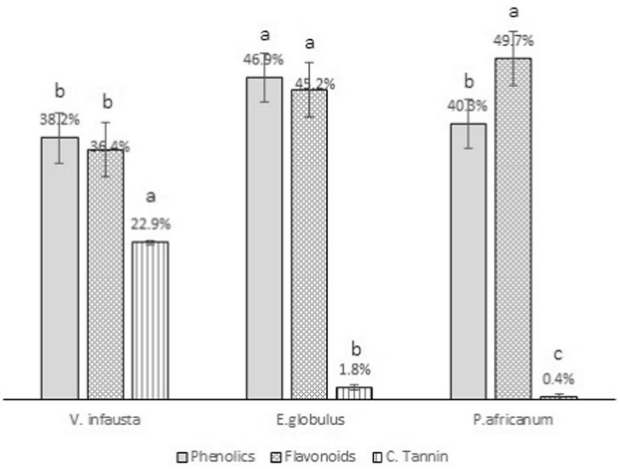

Fig. 1 Proportion of secondary metabolites measured in plant extracts of *Eucalyptus globulus, Peltophorum africanum* and *Vangueria infausta*

of proportion (Fig. 1), phenolics represented higher ($P < 0.05$) proportion in *E. globulus* (46.9%) than *V. infausta* (38.2%) and *P. africanum* (40.3%). Flavonoids and total phenolics were higher ($P < 0.05$) in both *E. globulus* and *P africanum* than *V. infausta*. Condensed tannins were significantly higher ($P < 0.05$) in *V. infausta* than *E. globulus* and *P. africanum.*

3.4 Antioxidant activity

The scavenging effects of plant extracts on DPPH is presented in Fig. 2 and radical scavenging activities (EC_{50}) in Fig. 3. All the results for antioxidant activity were dose-dependent. The effects of *V. infausta* and *E. globulus* increased with increasing concentrations from 0.3 to 25 µg/mL. From 25 to 200 µg/mL, there was a slight decrease in scavenging effects. These effects were similar ($P > 0.05$) for *V. infausta* and *E. globulus* extracts at all concentrations. No antioxidant activity was observed for extract of *P. africanum* at concentrations ≤ 12.5 µg/mL. Activities for *P. africanum* were evident at 25 µg/mL but were lower ($P < 0.05$) than the two other plant extracts at concentrations below 200 µg/mL. At 200 µg/mL the scavenging effects were similar ($P > 0.05$) to *V. infausta* and *E. globulus*. The EC_{50} values of scavenging DPPH radicals for *V. infausta* and *E. globulus* were similar averaging 9.3 µg/mL and 12 times lower than *P. africanum* (Fig. 3). When compared to ascorbic acid, values of scavenging DPPH radicals for *V. infausta* and *E. globulus* were 6 times greater ($P > 0.05$).

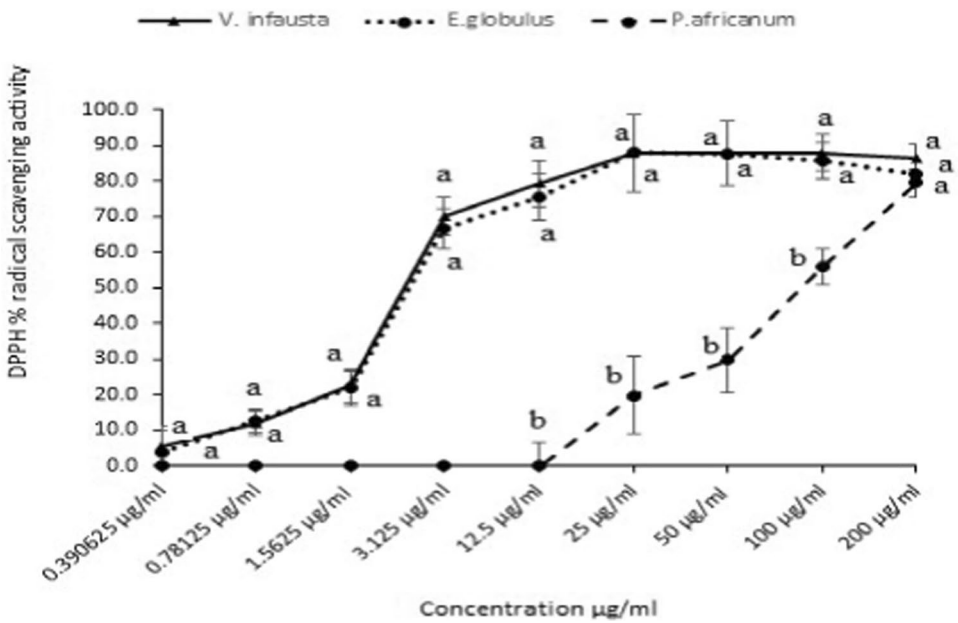

Fig. 2 Antioxidant activities of leaves extracts of *V. infausta, E. globulus and P. africanum:* diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

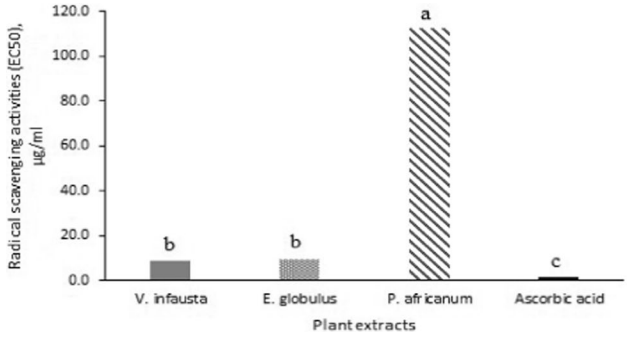

Fig. 3 Radical scavenging activities (EC50) of leaves extracts of *V. infausta, E. globulus* and *P. africanum* at different concentrations

3.5 Cytotoxicity

The evaluation of cytotoxic on Vero cell lines by XTT assay resulted in IC_{50} values ranged from 325.8 to > 400 µg/mL for all tested plant extracts, which were higher ($P < 0.05$) than that of the positive control (Table 4). The IC_{50} values of crude extract and essential oil (EO) from leaves of *E. globulus* were similar, 325 and 358 µg/mL, respectively.

3.6 Isolated bacteria

Eight bacterial species were isolated from the rumen liquor of the lactating Holstein dairy cows and identified as gram positive and gram negative bacteria (Table 5).

3.7 Antibacterial activity of *Eucalyptus globulus, Peltophorum africanum* and *Vangueria infausta* extracts

The MIC values of tested plant extracts for antibacterial activity are presented in Table 6. The antibacterial activity of leaves extract was lower ($P < 0.05$) for *P. africanum* and *E. globulus* oil, ranging from 3.12 to 12.5 mg/mL. Crude extracts of *E. globulus* and *V. infausta* showed the highest ($P < 0.05$) antimicrobial activity ranging between 0.19 and 1.56 mg/mL. A wider margin of effects was observed with extracts of *P. africanum*; a lean range was observed for gram-positive bacteria (6.25–12.5 mg/mL) compared to gram-negative bacteria (3.12 to 12.5 mg/mL). However, *Acidaminococcus fermentans* and *Klebsiella variicola* bacteria showed no activity in *E. globulus* essential oil extract. *E globulus* extract showed higher antimicrobial activity compared to the *E. globulus* oil.

Table 4 Cytotoxicity (µg/mL) results of *Eucalyptus globulus, Peltophorum africanum* and *Vangueria infausta* on Vero cell line

	E. globulus (CE)	*E. globulus* (EO)	*P. africanum* (CE)	*V. infausta* (CE)	Actinomycin D[a]	SEM	P-value
IC_{50} (µg/mL)	357.8	325.4	> 400	> 400	< 0.005	20.9	0.02
Toxicity	NT	NT	NT	NT	T		

Concentration of test sample to inhibit Vero cell line by 50%

[a]Positive drug control

NT not toxic, *T* toxic, *EO* essential oil, *CE* crude extract

Table 5 Bacteria isolated from the rumen liquor of Holstein lactating cow

Gram + ve	Gram –ve
Enterococcus vlikiensis	*Enterobacter cloacae*
Streptococcus lutetiensis	*Acidaminococcus fermentans*
Sharpea azabuensis	*Klebsiella variicola*
Enterococcus harae	*Escherichia coli*

Table 6 Antibacterial activity of plant extracts minimum inhibitory concentration (MIC) mg/mL of crude extracts of *V. infausta, E globulus*, and *P. africanum* and *E. globulus* essential oil

Gram + ve	Tested plant (leaves)			
	Vangueria infausta (CE)	*Eucalyptus globulus* (CE)	*Peltophorum africanum* (CE)	*Eucalyptus globulus* (EO)
E. vlikiensis	0.78	0.39	6.25	6.25
S. lutetiensis	0.39	0.19	12.5	12.5
S. azabuensis	0.78	0.39	12.5	6.25
E. harae	1.56	1.56	12.5	6.25
Gram -ve				
E. cloacae	0.78	0.19	3.12	12.5
A. fermentans	1.56	0.19	12.5	Na
K. variicola	1.56	0.78	12.5	Na
E. coli	0.78	0.78	12.5	3.12

Na not active, *CE* crude extract, *EO* essential oil

4 Discussion

4.1 Chemical composition and extract yield

The yield and composition of EO is related to the physiological development of plants and represent a chemical interface between the plants and the environment [58] however, its synthesis is often affected by environmental conditions [59]. Since all three plant leaves where harvested from the same environment and at the same time, although their genetic make-up is different, it is possible that EOs in *E. globulus* play an important role in the plant defence compared to *P. africanum* and *V. infausta* no wonder the high yields in the current study, similar conclusions were suggested by Castelo et al. [58]. It can also be that *E. globulus* is more affected by external conditions, making it produce more EOs. This would justify the commercial cost of extracting the oil of *E. globulus* and its use as additives in large animal research such as high producing dairy cows.

4.2 Secondary metabolites

The phytochemical content of plant extracts, in the current study, and their antioxidant effects are presumptive of their use as animal feed supplements for improved animal health and subsequent performance. Although both plant extracts and EOs are a source of biologically active compounds and natural antioxidants [60–62], EOs have higher concentration of these compounds, therefore, are required in much lower quantities or dosage compared to crude extracts [63, 64]. The total phenolics and flavonoids content of extract from *E. globulus* in this study were comparable to the average value of 227.3 mg GAE/g and 192.5 mg QE/g reported by Luís et al. [65], but phenolics were much more than 62.10 mg GAE/g reported by Mocan et al. [66]. A number of factors, including plant species, genetic factors, geographical location, and type of soil, season of harvesting, herb preparation, drying and storage [66] regulate the presence of biologically active compounds consequently determining their concentration in plants. Some of these conditions might have contributed to the concentration observed in the present study. The elevated level of phenolics, condensed tannins, and flavonoids in *V. infausta* [34, 35, 62, 67] and *E. globulus* [68] have been reported. Therefore, *V. infausta* and *E. globulus* are preferred as choice plants for compound extraction purification and eventual commercialisation in the current study. Flavonoids and other phenolic compounds have been reported for their effective antioxidants, antibacterial and immune system properties amongst other bioactivities [69–71]. These two compounds are considered an interesting choice of molecules for medical product development [38] and can be useful for development of ruminant feed additives due to their reported antibacterial activities similar to ionophores [72]. The presence and magnitude of condensed tannins (CTs) in the current study is commendable from an animal perspective, since they significantly affect ruminal fermentation and has been associated with protein degradation and decreased methanogens [73, 74]. The decreased methanogens are known to improve animal

performance by sparing energy [75–77]. Phenolics and flavonoids were the most abundant compounds in all the extract and CT in *V. infausta* making the three compounds a basis for extract selection for the purpose of improving rumen fermentation. However, the highest concentrations of phenolics and flavonoid associated with the highest oil yield in *E. globulus* leaves extract suggest the later extract is more suitable to be tested and used as additives for the purpose of improving rumen fermentation as an alternative to antibiotics.

4.3 Antioxidants activity

The highest capacity to neutralize DPPH radicals was found in *V. infausta*, and *E. globulus* extracts, thus they had much more hydrogen donating ability or radical scavenging activity (61, 78, 79) and, the ability to inhibit the autoxidation of lipids and delay lipid oxidation by inhibiting lipoxygenase activity, preventing cell damage [80]. In comparison to antioxidant activity of pure standard antioxidants (Vitamin C), extracts of *V. infausta*, and *E. globulus* were 12 times less powerful to scavenge DPPH radicals [81], thus the antioxidant activity of the two plant extracts could be considered as strong and can be used as supplement antioxidants in animal feeding. The higher antioxidant activity in the current study is attributed to the higher phenolic and flavonoid content of the plant extracts. This has been observed by Nunes et al. [82], who concluded that the inhibition of lipid peroxidation increased with increasing concentrations of phenolics and flavonoids. Thus there is a strong positive correlation between the antioxidant effects and the phenolics and flavonoid content [61], therefore, can be used as the main determinants in evaluating antioxidant potential of plants [83]. Although saponins have been reported to possess radical scavenging activity potential [83, 84], their level (9.3–10.5%) in the present study may be limited to effect antioxidant effects. In a study by Chan [85], saponin levels ranging from 114.22 to 190.51 mg DE/g were considered antioxidant. The present results suggested that phenolic acids and flavonoids level in *V. infausta,* and *E. globulus* may be the major contributors for the antioxidant activity as the EC_{50} values of radical scavenging activity could play a beneficial role in improving animal health. This antioxidant activity potential could explain the beneficial effect of parts of these plants in the traditional treatment of a range of diseases in humans [86].

4.4 Cytotoxicity

In the present study, following 72 h of incubation, all tested plant extracts had IC_{50} values higher than 300 µg/mL, which suggested that they were all non-cytotoxic [87] compared to the control. Therefore, crude extracts *V. infausta, E. globulus, P. africanum*, and EO of *E. globulus* may be suggested to be safe for use in animals. Most of the studies that revealed cytotoxicity of plant extracts from *E. globulus* [88–90] and many other EOs on cancer cell lines showed little damage or non-toxicity on normal cells [27, 91], suggesting selective activity. In several previous studies, extracts from leaves of *Eucalyptus* species showed some degree of cytotoxicity against human cancer cell lines [88, 91–94]. Kumari and Jesudas [95] reported cytotoxicity effect of methanol extract of *E. globulus* on breast cancer cell lines (IC_{50} = 145.8 µg/mL), Doll-Boscardin [89] observed a cytotoxicity of EO from leaves of *E. benthamii* on cancer cells lines. The range of IC_{50} values observed in these studies (9.59 to 145.8 µg/mL) is much lower than values observed in our study, suggesting that the extracts used in the present study are safe. The cytotoxic effects are attributed to the lipophilic nature and low molecular weights of the constituents of EOs [96], for example, the hydrophobic nature of EOs allow them to penetrate cell membranes, altering the phospholipid layers, increasing membrane fluidity, and leading to leakage of ions and of cytoplasmic content [97]. Results from Aldaghi [98] confirm that the plant extracts as well as their compounds could kill cancer cells with little damage to normal cells and hence were selectively active. Phenolic compounds commonly have protective and deleterious effects that are dose-depended [21], and toxicity of plant extracts is associated to wrong use causing accumulation of potentially toxic constituents or interactions with other products [99]. It is therefore important to ensure that the dose is maintained below toxicity threshold suggested in the present study.

4.5 Antibacterial activity of *V. infausta, P. africanum and E. globulus*

Antibacterial activity is higher for EOs compared to crude extract in the current study, similar results have been reported by Salem et al. [20] in which the MIC was lower for EO than water extract (WE) of *Schinus molle*. Furthermore, Domenech et al. [100] reported MIC values of 160–300 µg/mL to both *Streptococcus pneumoniae* and *Haemophilus influenza*. Essential oils induce damage in the mitochondrial membrane structure by decreasing the membrane potential and altering the fluidity of membranes permanently [6]. In addition, the ability of flavonoids to pass through the lipopolysaccharide layer of gram + ve and not gram −ve bacteria [72] is consequential. This resistance is explained by the complexity of gram + ve

bacteria's cell membrane. However, this property has a positive implication in improving ruminant energy balance, making plant extracts a good alternative to antibiotics use in animal production. Furthermore, saponins acted on gram + ve bacteria and the results from the current study confirm the presence of elevated levels of saponins in *E. globulus*. The selective antimicrobial activity of saponins has been reported [101], saponins act by the formation of complexes with cholesterol in the cell membrane resulting in the gram + ve cell lysis [72]. Thus, the presence of flavonoids and saponins in the plants extracts was the reason for antimicrobial capability in the current study. The change in rumen bacteria population has significant effects on rumen fermentation and consequently gas production and emission [102], a modern topical issue. The addition of plant extracts from *E. globulus*, *P. africanum* and *V. infausta* have the potential to modulate rumen characteristics and consequently the by-products of fermentation.

5 Conclusions

Leaves of *E. globulus* yielded more crude extract and EO, which could justify its use in large animal feeding such as dairy cows. The leaves extracts may benefit livestock as feed supplements and serve as free radical inhibitors, scavengers, or acting as primary antioxidants. The high antioxidant capacity for *V. infausta* and *E. globulus* are associated with their phenolics and flavonoids content, and should be perused to produce antioxidant additives for livestock feeding. All the tested plant extracts were non-toxic at a concentration of 0.3 mg/mL. In addition, these plant extracts have a great potential as antimicrobial compounds and feed additives for increased ruminant production. The purification of active compounds in *V. infausta* and *E. globulus* may lead to drug development, thus ease on drug resistance by microorganisms and is therefore recommended for future research.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and preparation were performed by Portia Mamothaladi Moshidi experimental design and statistical analysis was done by Soul Washaya. The first draft of the manuscript was written by Portia Mamothaladi Moshidi, Johnfisher Mupangwa and Claude Mukengela Muya supervised the project and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Data will be made available upon reasonable request from the corresponding author.

Declarations

Ethics approval and consent to participate The experimental protocol and procedures were approved by the Animal Ethics Committee (MUP141SMOS01) of the University of Fort Hare, Eastern Cape, South Africa. All animal experimental methods were carried out in accordance with the ARRIVE guidelines and regulations. Consent to harvest plant materials from the wild was obtained from local authorities.

Competing interests The authors declare no competing interests.

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