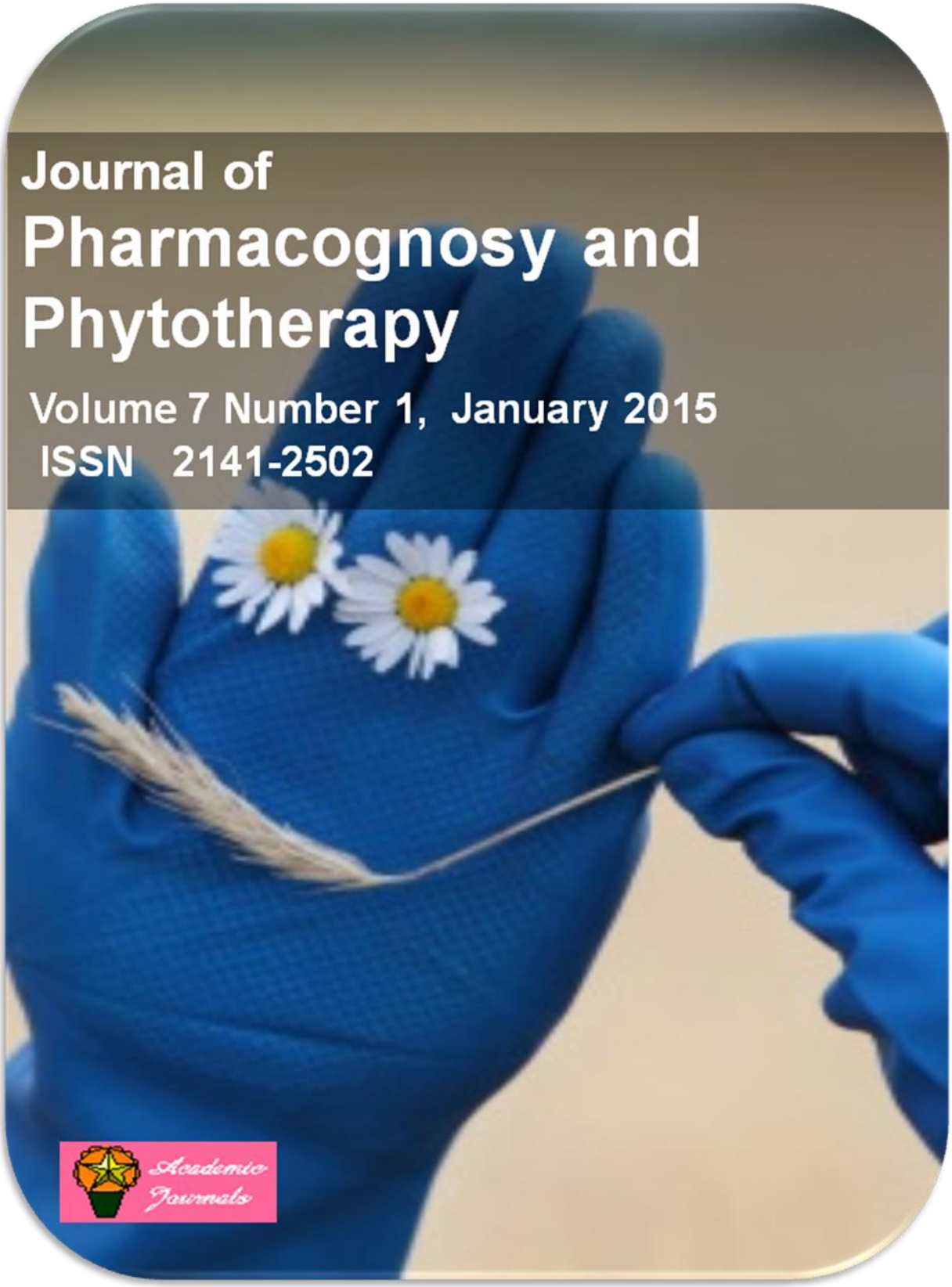
Journal of Pharmacognosy and Phytotherapy

Volume 7 Number 1, January 2015
ISSN 2141-2502



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Journal of Pharmacognosy and Phytotherapy

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Research Article

academicJournals

Vol. 7(1), pp. 1-8, January 2015
DOI: 10.5897/JPP2014.0336
Article Number: 5E9171750300
ISSN 2141-2502

http://www.academicjournals.org/JPP

Journal of Pharmacognosy and Phytotherapy

Full Length Research Paper

Pharmacological properties of *Pomaria sandersonii, Pentanisia prunelloides* and *Alepidea amatymbica* extracts using *in vitro* assays

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Received 17 October, 2014; Accepted 31 January, 2015

The antimicrobial, anti-inflammatory and free radical scavenging activities of root crude acetone extracts and fractions of different polarities from *Pomaria sandersonii* (Fabaceae), *Pentanisia prunelloides* (Rubiaceae) and *Alepidea amatymbica* (Apiaceae) were determined using *in vitro* assays. The antioxidant properties of extracts and fractions were assessed by reduction of 2, 2´-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid and 2, 2-di (4-*tert*-octylphenyl)-1-picrylhydrazyl radicals which was measured by changes in absorbance using an ultraviolet-visible spectrophotometer. Anti-inflammatory activity of the plant extracts against 15 soybean lipoxygenase enzyme was measured by monitoring the change in absorbance at 234 nm after incubation of 15-LOX with linoleic acid (134 µM) as substrate. The anti-microbial activities were determined by measuring the minimum inhibitory concentrations using a serial dilution microplate method with terazolium violet as a growth indicator. The minimal inhibitory concentration (MIC) value of the dichloromethane (DCM) and ethyl acetate fractions (1 mg/ml) of *P. sandersonii* was 80 µg/ml in each case against *Staphylococcus aureus* and *Escherichia coli*. The inhibition activity of 15 soybean lipoxygenase enzyme by the crude extracts at concentration of 25 µg/ml was 97% for *P. sandersonii*, 79% for *P. prunelloides* and 55% for *A. amatymbica.* This indicates that extracts for these plants can be used as dietary supplements in the management of inflammation related conditions.

Key words: Extract, fraction, anti-inflammatory, antibacterial, anti-oxidant, antifungal, antioxidant.

INTRODUCTION

The pathogenic bacteria associated with some diseases in many communities/settlements in South Africa include *Escherichia coli* (gastroenteritis); *Enterococcus faecalis* (endocarditis), *Pseudomonas aeruginosa* (inflammation, sepsis in the lungs, urinary tract and kidneys) and *Staphylococcus aureus* (tonsillitis, scarlet fever, minor skin infections, impetigo, boils, abscesses and scalded skin syndrome) (Hamer, 2007). Some of the fungal pathogens also associated with diseases in the community include *Candida albicans,* which are important

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opportunistic yeast involved in oropharyngeal and genital candidiasis (Pfaller and Diekemer, 2004). *Aspergillus fumigatus* is implicated in causing a range of diseases called aspergillosis whose symptoms include cough, fever, wheezing, skin sore and vision problems (Walsh et al., 2008).

In Zululand of South Africa, the use of medicinal plants for prevention and cure of different ailments is well established as part of their cultural heritage. *Alepidea amatymbica* Eckl. & Zeyh. (Apiaceae) is one of the most important species used as medicinal plant in KwaZulu-Natal (KZN) of South Africa and Lesotho (van Wyk, 2008). The rhizomes and roots of *A. amatymbica* (known as Ikhathazo) are used for the treatment of colds and chest complaints (Watt and Breyer, 1962). The plant is used in treating gastrointestinal disorder, respiratory tract infection, hypertension and constipation (Hutchings, 1989). The antimicrobial, cyclooxygenase-1 and 2, genotoxicity (Mulaudzi et al., 2009) and antiplasmodial activity (Clarkson et al., 2004) of *A. amatymbica* root extracts have been reported before. Phytochemical investigation of the root and aerial part of the plant revealed the presence of ent-9, (11)-dehydro-16-kauren-19-oic acid, ent-16-kauren-19-oic acid (Rustaiyan and Sadjadi, 1987) which may be the active compounds. Kaurenoic acid isolated from *Helichrysum krausii* is active against *S. aureus*, *Bacillus cereus*, *Bacillus subtilis* (MIC 10 µg/ml), *Escherichia coli* and *S. marcescens* (1 to 10 µg/ml) (Bremner and Meyer, 2000). However, there is no report on antioxidant activities of extracts and fractions from *A. amatymbica.*

Pentanisia prunelloides (Kotzeh ex Eckl & Zeyh.) Walp. (Rubiaceae) also features as a prominent medicinal plant for treating ailment associated with inflammation, microbial infection, muscular contraction stomach pain haemorrhoids, snakebite and rheumatism in Zulu traditional medicine (Hutchings et al., 1996). Boiled grated dried bulb is usually taken orally to stop vomiting and diarrhoea in children (Bisi-Johnson et al., 2009). Anti-microbial, cytotoxicity and cyclooxygenase-1 enzyme inhibitory activity of the plant has been reported (Jager et al., 1996). Root and leaf extracts of *P. prunelloides* inhibit COX-1 and the viral replication of the influenza A virus (Yff et al., 2002). Pharmacological investigation of the plant has led to the isolation of palmitic acid as the anti-microbial agent (Yff et al., 2002). *Pomaria sandersonii* (Fabacea) (Harv) B. B. Simpson and G. P. Lewis is also used in Zulu traditional medicine for pain, inflammation and anti-haemolytic activity (Dlamini personal communi-cation). It is endemic to South Africa, grows in the Eastern Cape, KwaZulu- Natal region. It is on the red list of South African plants (Raimondo et al., 2009). This is the first time of recording the ethno pharmacological use of the plant in southern Africa. There is no scientific report on the biological activities of extracts from *P. sandersonii*.

The aim of this study was to scientifically validate the traditional use of *P. prunelloides* (Rubiaceae), *P. sandersonii* (Fabaceae) and *A. amatymbica* (Apiaceae) in treating infections and oxidative stress relating to inflammation. Therefore, this work focuses on the antibacterial, antifungal, antioxidant (DPPH and ABTS radical scavenging assays) and anti-inflammatory activity of the crude extracts and their fractions of different polarities.

MATERIALS AND METHODS

Plant selection and collection

An ethnobotanical survey based on verbal interviews conducted with nine traditional healers of Mabandla village of Umzimkhulu Local Municipality, Kwa-Zulu Natal, South Africa (30° 15′ 45″ S, 29° 55′ 15″ E) for plants used traditionally in the treatment of infectious and inflammatory diseases was carried out. The plants listed in Table 1 were identified and collected with the help of Mr. Sanoyi Paulos Dlamini the head of traditional healers from the village. Authentication of plants was done at South Africa National Biodiversity Institute, Pretoria and their voucher specimens are maintained at Pretoria National Botanical Garden.

Plant treatment

Plant root and bulb materials collected from Mabandla village, Kwa-Zulu Natal, were washed, air dried at room temperature for three weeks and ground to powder using a Lasec Polymix PX-MFC 90D grinder. Dried plant pulverised material was stored in glass containers in a cool dry place. Crude extracts were made by shaking fine ground plant powder (200 g) in a ratio of 1 g to 10 ml acetone for 6 h followed by filtering (Eloff, 1998a). Excess solvent was recovered on a rotary vapour until the extract was concentrated. Concentrated slurry was dried at room temperature in the fume hood under an air stream. The crude dried plant extract was stored at 4°C until it is required for biological assays.

Liquid-liquid extraction (fractionation)

Dried crude acetone extracts were re-constituted in 200 ml of 70% acetone and extracted sequentially with hexane followed by dichloromethane, ethyl acetate, acetone and methane. The residual water fraction was dried in a conventional oven at 50°C for 96 h. Absolute methanol was added every 24 h to prevent the growth of fungi. The dried water fractions were extracted with acetone and methanol successively to produce fractions of different polarities. Filtered fractions were concentrated on a rotary evaporator and then air dried at room temperature under a fan. The dried fractions were weighed and results are recorded in Table 2.

Quantitative evaluation of the biological activities of the plant extracts

Minimum inhibitory concentrations (MIC)

Minimum inhibitory concentrations of the crude extracts and fractions were determined by twofold serial dilution using 96-well microtitre plate against *S. aureus* (ATCC 29213), *P. aeruginosa* (ATCC 27853), *E. faecalis* (ATCC 29212) and *E. coli* (ATCC 25922). Tetrazolium violet was used as a growth indicator (Eloff, 1998b). In brief, 100 µL distilled water was placed in each of the wells using a multichannel micropipette. Thereafter, 100 µL of extract (10 mg/ ml) was added to the first well of column and serially diluted to prepare

Table 1. Ethnopharmacological information of the plant use in this study.

Botanical name/traditional names	Voucher number	Traditional medicinal use
Alepidea amatymbica Eckl. & Zeyh (Apiaceae) (Ikhathazo(isiZulu) Losoko,(Sesotho)	2116-0	Colds, influenza, stomach and respiratory ailments, rheumatism and wounds (Somova et al., 2001; Stafford et al., 2004; Van Staden et al., 2009).
Pentanisia prunelloides, Klotzeh ex Eckl & Zeyh.) Walp. (Rubiaceae) (Isicimamlilo (isiZulu)	1200-1	Root extract is used to treat aches and pains Kaidoo, T. L. (1997)
Pomaria sandersonii (P.s.) (Harv) B. B. Simpson & G.P. Lewis (Fabaceae) (Istholwane (isiZulu)	14806-0	Root extract is used for post natal care for mothers after child birth for fast recovery Traditional healer, Mabandla Village, Umzimkulu, Kwa-Zulu Natal, South Africa (2009).

Table 2. The percentage yield of the crude extracts and fractions of the plants**.**

Plant species	Crude	H	DCM	ET	AC	METH	W
P. sandersonii	7.818±0.78	0.64±0.06	31.33±3.10	16.34±1.60	23.77±2.20	25.18±2.50	-
A. amatymbica	7.65±0.76	31.14±3.10	41.31±4.10	0.91±0.09	0.84±0.08	23.73±2.37	-
P. prunelloides	5.05±0.49	1.15±0.12	0.82±0.08	-	9.83±0.10	7.05±.08	80.81±8.00

C= crude extract, H= hexane fraction, DCM= dichloromethane fraction, ET = ethyl acetate fraction, AC = acetone fraction and METH= methanol fraction, W= Water fraction.

a concentration range between 5.0 and 0.04 mg/ml in the first well and last well respectively. The solvent blank (negative control) and antibiotic (gentamicin for bacteria and amphotericin B for fungi) were included as positive control. From an overnight culture of bacteria grown in diluted Mueller Hinton broth, 100 µL of relevant bacteria dispensed into each well and incubated at 37°C for 18 to 24 h before adding 40 µl (0.2 mg/ml) of iodonitrotetrazolium violet, Sigma (INT) solution. Colour change was noted after 30, 60, and 120 min to determine the lowest concentration which inhibited growth (MIC). MIC of the crude extracts and fractions against *Candida albicans* and *A. fumigatus* were determined using the above-mentioned serial dilution assay with some modifications (Masoko et al., 2005). Fungal stock was prepared using Sabouraud dextrose broth instead of Mueller Hinton broth used for bacteria culture. All measurements were performed in triplicate.

DPPH anti-oxidant assay

For DPPH anti-oxidant assay, using 96 well plates and a VERSAmax^{TM} tunable microplate reader (Labotech), 40 µl of 0,5 mg/ml plant extract and fraction were determined by a twofold serial dilution in 160 µl of 0.0025% of DPPH (total volume 200 µl), methanol (negative control) or trolox (positive control). After 30 min, the absorbance was measured at 516 nm wavelength. Methanol was used as negative control and trolox (2.5 to 0.5 mg/ml) was used as positive control. The free radical DPPH$^{\cdot}$ scavenging (reduction) was calculated from the equation: Activity (% of DPPH reduction) = $\{(A - A_x) / A\} \times 100\%$, where A is absorbance of DPPH solution with methanol, A_x is absorbance of a DPPH solution with a tested fraction solution or trolox (positive control). All measurements were performed in triplicate.

ABTS$^{\bullet+}$ Scavenging assay

The ABTS$^{\bullet+}$ radical cation was prepared by mixing 7 mM ABTS stock solution and incubating for 12 to16 h in the dark at room temperature until a steady absorbance was obtained to indicate that the reaction was complete. Plant extracts and fractions (40 µL of 0.5 mg/ml) were mixed with 160 µL of the ABTS$^{\bullet+}$ radical cation (total volume 200 µl) in a 96-well microtitre plate. Absorbance of the mixture was read at 734 after six minutes using VERSAmax^{TM} tunable microplate reader (Labotech). Methanol was used as negative control and trolox (25 to 0.5 g/ml) was used as positive control. All measurements were performed in triplicate.

Lipoxygenase inhibition

Inhibitory activity of the plant extracts against 15-soybean lipoxygenase (15-LOX) enzyme was evaluated according to Malterud and Rydland (2000) in borate buffer (0.2 M, pH 9.00). Increase in absorbance at 234 nm was read 5 min at interval of 30 s after addition of 15-LOX, using linoleic acid (134 µM) as substrate. The final enzyme concentration was 167 µg/ml. Test substances were added as dimethyl sulfoxide (DMSO) solutions (final DMSO concentration of 1.6%) while DMSO alone was added in control experiments. The enzyme solution was kept on ice, and controls were measured at intervals throughout the experimental periods to ensure that the enzyme activity was constant. All measurements were performed in triplicate.

RESULTS

Yield

The yields of the crude methanol extracts and fractions of various polarities were presented in Table 2. The highest percentage yield of 7.82% was from *P. sandersonii* but had the lowest hexane fraction yield of 0.64%. *A. amatymbica* extract had the highest fraction of non-polar

Table 3. Free radical scavenging activity of the crude extract and fractions of varied polarities (µg/ml).

Sample	*Pomaria sandersonii*		*Alepidea amatymbica*		*Pentanisia prunelloides*	
	DPPH (EC_{50})	ABTS (EC_{50})	DPPH (EC_{50})	ABTS (EC_{50})	DPPH (EC_{50})	ABTS (EC_{50})
Cr	5.6±0.71	3.24±0.13	7.68±0.59	1.27±0.16	68.89±9.21	213.66±35.00
H	3.58±0.44	11.31±3.70	19.82±1.74	152.00±22.45	111.93±10.55	3987.33±372.75
DCM	2.93±0.14	7329±117.53	36.25±3.33	1.75±1.02	98.62±6.55	28.68±9.46
ET	-	88.76±3.50	13.51±1.03	-	12.49±0.42	1.10±0.26
Ac	70.62±5.38	102.40±15.44	4.17±0.37	5.96±0.82	5.36±1.05	0.08±0.03
Met	2.61±0.07	0.19±0.04	31.34±2.00	-	4.08±0.65	2.72±1.77

Cr = crude extract, H = hexane fraction, DCM = dichloromethane fraction, ET = ethyl acetate fraction, Ac = acetone fraction, Met = methanol fraction, Positive standards: trolox = 0.04±0.001, ascorbic acid = 0.04±0.001

constituents (72%) that were hexane and DCM soluble. *P. prunelloides* had the highest water soluble component of 80% yield.

Antimicrobial activities

The minimum inhibitory concentrations of the crude methanol extracts and fractions of various polarities were presented in Figures 2 to 4. The antimicrobial activities of *A. amatymbica* crude extract and fractions were moderate to good against the bacterial and fungi tested with MICs ranging from 160 to 625 µg /ml. The crude extracts and fractions of *P. prunelloides* had weak (160 to 320 µg /ml) antibacterial activity (Figure 4). The anti-fungal activity was low at 625 µg /ml for the crude extract and fractions from *P. prunelloides*. The crude extract and fractions of *P. sandersonii* displayed good antimicrobial activity with MICs ranging from 20 to 1250 µg /ml (Figure 3). Dichloromethane and ethyl acetate fractions of *P. sandersonii* had MIC of 80 µg /ml against *S. aureus, E. coli* and *P. aeruginosa* which is comparable to the activities of the antibiotic standard gentamycin. The fractions had good activity against *E. faecalis* with MIC of 40 µg /ml. The dichloromethane, acetone and methanol fractions of the *P. sandersonii* also had good antifungal inhibitory activity (20 µg /ml) against C*. albicans* and *A. fumigatus* (Figure 3).

Antioxidant and lipoxygenase inhibitory activity

Pentanisia prunelloides

The EC_{50} values for hexane, DCM and acetone *P. prunelloides* fractions indicated that they were more reactive with $DPPH^{\bullet}$ radical while there was a comparable activity with both radicals with the methanol fractions with EC_{50} of 2.619 µg/ml for $DPPH^{\bullet}$ and µg/ml for $ABTS^{\bullet+}$ radicals (Table 3). The lipoxygenase inhibitory activity of 79% indicates high anti-inflammatory activity.

Pomaria sandersonii

All the plant extracts and fractions used in this study displayed some anti-radiative activity although, the crude extract $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ for *P. sandersonii* displayed the highest activity compared to all of the plant's samples. DCM fraction was more reactive towards $ABTS^{\bullet+}$ displaying activity of 0.19 µg/ml compared to the methanol extract which had $DPPH^{\bullet}$ displaying activity of 2.61 µg/ml (Table 3). The hexane fraction was more active against $DPPH^{\bullet}$ compared to the reaction with $ABTS^{\bullet+}$ radical. The crude extract of *P. sandersonii* had a high lipoxygenase inhibitory activity of 97% at concentration of 25 µg/ml.

Alepidea amatymbica

The DCM fractions of *A. amatymbica* also displayed different activities towards the two radicals. EC_{50} values for the crude and acetone fractions were 4.17µg/ml towards $DPPH^{\bullet}$ and 5.96µg/ml towards $ABTS^{\bullet+}$ respectively. The lipoxygenase inhibitory activity of 55% at concentration of 25 µg/ml indicated moderate anti-inflammatory activity for *A. amatymbica*. The results indicate that the *A. amatymbica* fractions contain antioxidants.

DISCUSSION

Our results demonstrated biological activities of the methanolic crude extracts, hexane, dichloromethane, ethyl acetate and acetone fractions obtained from *A. amatymbica, P. prunelloides* and *P. sandersonii* which are used to manage inflammation related conditions. The plants exhibited antibacterial, anti-inflammatory and anti-oxidative properties.

The antimicrobial activity (MIC) of the extracts was determined using the broth microdilution method based on the principle of contact of a test organism to a series of dilutions of test substance. The crude extract and fractions (hexane, dichloromethane, ethyl acetate) of *A. amatymbica* (Figure 1) displayed good to moderate (160 to 320 µg/ml) anti-bacterial activity against all the organisms tested, however, the methanol fraction

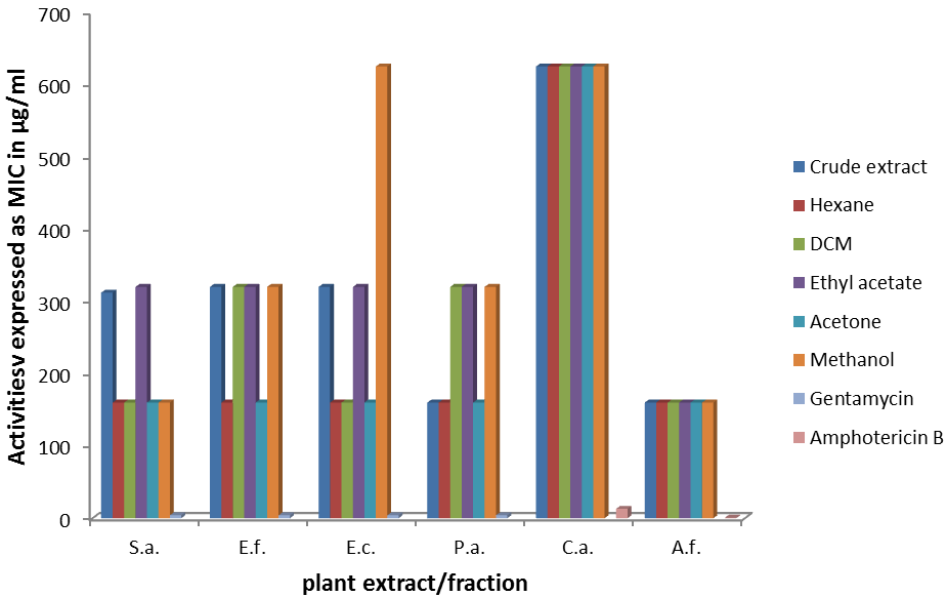

Figure 1. Antimicrobial activities of *Alepedia amatymbica* crude extracts and fractions of different polarities as indicated by solvent used: S.a .= *Staphylococcus aureus*, E.f .= *Enterococcus faecalis*, E.c. = *Escherichia coli*, P.a. = *Pseudomonas aeruginosa*, C.a. = *Candida albicans*, A.f .= *Aspergillus fumigatus.* Gentamycin and amphotericin B were the positive standards.

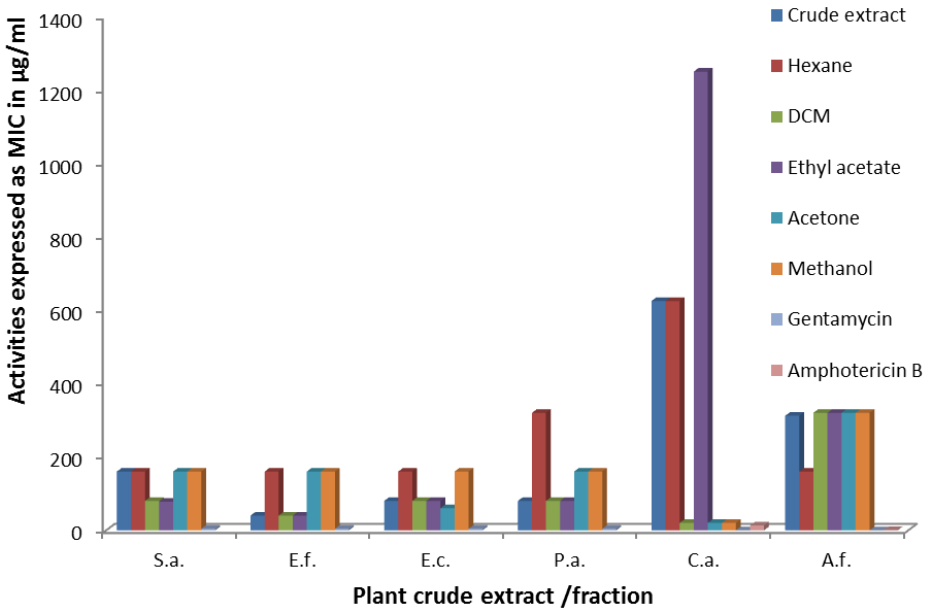

Figure 2. Antimicrobial activities of *Pomaria sandesonni* crude extracts and fractions of different polarities as indicated by solvent used: S.a .= *Staphylococcus aureus*, E.f .= *Enterococcus faecalis*, E.c. = *Escherichia coli*, P.a. = *Pseudomonas aeruginosa*, C.a. = *Candida albicans*, A.f .= *Aspergillus fumigatus.* Gentamycin and amphotericin B were the positive standards.

showed a lower MIC (625 µg/ml) against *E coli*. The activity of the *A. amatymbica* extract and fractions against *C. albicans* was also low (MIC = 625 µg/ml). In a related study, Mulaudzi et al. (2009) reported lower anti-bacterial and anti-fungal activities of polar root extracts and fractions (water and ethanol) from *A. amatymbica.* The

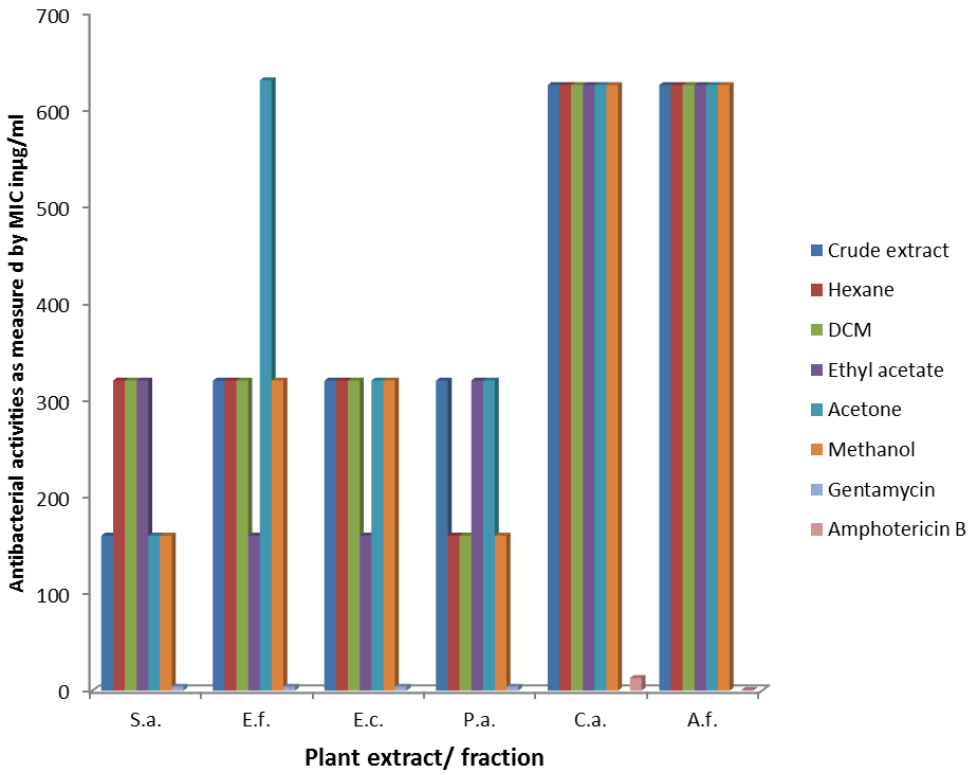

Figure 3. Antimicrobial activities of *Pentanisia prunelloides* crude extracts and fractions of different polarities as indicated by solvent used: *S.a .= Staphylococcus aureus*, *E.f .= Enterococcus faecalis, E.c. = Escherichia coli, P.a. = Pseudomonas aeruginosa, C.a. = Candida albicans, A.f .= Aspergillus fumigatus.* Gentamycin and amphotericin B were the positive standards.

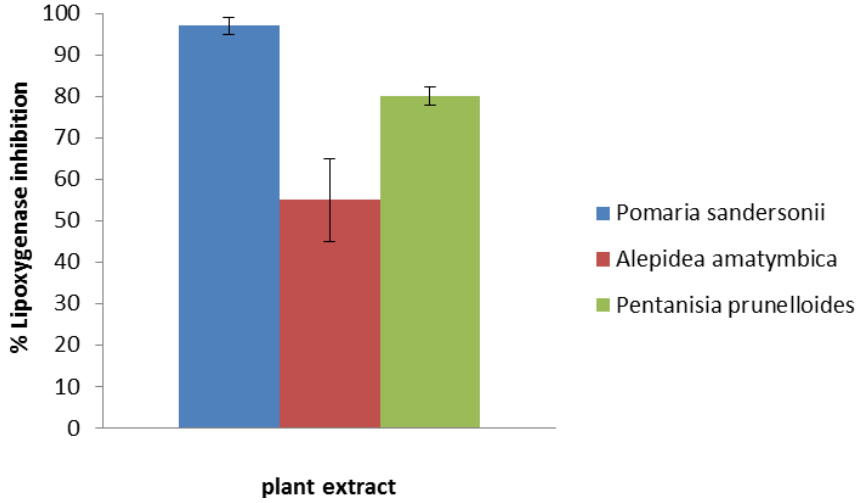

Figure 4. Percentage inhibition of 15-soybean lipoxygenase enzyme by the crude extracts at concentration of 25 µg/ml (n=3).

antimicrobial activity of *P. Prunelloides* (Figure 3), which is used traditionally for the treatment of dysmenorrhoea, was good to moderate (160 to 320 µg/ml). Anti-bacterial activity of the *P. prunelloides* acetone fraction was low displaying MIC of 625 µg/ml against *E. faecalis*. Yff et al. (2002), also reported a similar investigation, that anti-bacterial activity of the water, ethanol and ethyl acetate extracts from *P. prunelloides* root had low bacterial growth

inhibition (0.39 to 12.5 mg/ml). The low activity values in some of the extracts tested in this study could be due to the impure form and/ or low concentration of the active compound(s) in the extracts (Rabe and Van Staden, 1997). DCM and ethyl acetate fractions of *P. sandersonii* (Figure 2) displayed highest inhibitory activities (80 µg /ml in each case) against *S. aureus, E. faecalis, E. coli* and *P. aeruginosa* compared to the other two plants in this study. The more *p*olar fractions (acetone and methanol) had good anti-candidal activity (MIC= 20). The antimicrobial activity of the three plant extracts and fractions were more significant in the non-polar fractions of hexane and dichloromethane. The relatively higher inhibitory activities could be attributed to the presence of terpenoids which had been detected in crude extracts during an earlier qualitative test study, although the compounds have not been isolated and tested individually (Van Wyk , 2008).

Inflammation is an important process involved in the defence mechanisms of an organism against infectious and other deleterious stimuli. However, inflammatory responses to infectious agents can evade control by immuno-regulatory mechanisms. Existing treatment protocols for such inflammation-driven diseases remain less than optimal. In this study, ethanol crude extract of *A. amatymbica* rhizome had 50% LOX inhibition (Figure 4). Although, Mulaudzi et al. (2009) reported that the petroleum ether, DCM and ethanol extracts of *A. amatymbica* rhizome has 90% inhibitory COX-2 activity. The difference in activity could be due to the difference of LOX enzymes and methods used in determining the anti-inflammatory property of the plant. *P. prunelloides* had high 15-LOX inhibitory activity (79%) with EC_{50} value of 15.98 mg/L. The more polar leaf extracts of *P. prunelloides* earlier reported had good COX-1 inhibitory activity (leaf water extract = 74%, root water extract =74%) (Yff et al., 2002). Methanol crude extract of *P. sandersonii* was the most active with 97% LOX inhibitory activity. This is consistent with the fact that the fraction, being the most polar is also rich in polyphenols which are responsible for the anti-inflammatory activity.

Oxidative stress plays a significant role in the patho-genesis of infectious and inflammatory disease. The crude extracts, and more polar fractions of ethyl acetate, acetone and methanol soluble from the three plants have appreciable antioxidant activity. Acetone crude leaf extract, DCM and Ethyl acetate fractions of *P. sandersonii* had higher $ABTS^{\bullet+}$ scavenging ability compared to corresponding reaction with the DPPH radical. *P. sandersonii* samples (crude and fractions) were the most active against $ABTS^{\bullet+}$ (1.274 to 5.973 µg/ml) compared to all the plants studied (Table 3), except for the hexane fraction which displayed low activity (111.93µg/ml for DPPH) and (3987.33µg/ml for ABTS).

The low activity in the hexane fraction could attribute the possible absence of polyphenols in the non-polar fraction as compared to the high activities displayed by the more polar fractions rich in polyphenols. This is the first time reporting biological activities of *P. sandersonii* and are compared with the two relatively more well-known South African medicinal plants.

Conclusion

This study revealed that while medicinal plants still play a very vital role in the primary health care of the people of Mabandla village, Zululand, South Africa, the three plants in this study possess significant biological activities as indicated by the *in vitro* assays that were carried out. Taken together, the anti-infectious, anti-inflammatory and antioxidant mechanisms may help to develop better novel pharmacological drugs for various degenerative diseases. In this study, 15-LOX from soybean was used to assess the *in vitro* inhibitory activity. These findings should therefore be cautiously applied to the anti-inflammatory activity in humans because the mechanism of human derived 15 LOX may be different from that which is soybean derived. Other enzymes such as COX-1 and COX-2 should be studied to obtain a more accurate assessment of anti-inflammatory activity of the plants. These medicinal plants have demonstrated broad spectra of activity that might help to discover new pharmacological compounds which could serve as agents for human and animal health maintenance. The results obtained in this study also validate the traditional use of these plants to manage infectious and inflammation related disorders, however, *in vivo* studies are still necessary to completely validate the claims leading to isolation and characterisation of active compounds.

ACKNOWLEDGEMENTS

The authors acknowledge the University of Pretoria Phytomedicine programme, Vaal University of Technology for funding the study, Traditional healers of Mabandla Village, Kwa-Zulu Natal, South Africa and Midlands State University, Gweru Zimbabwe.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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