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In vitro antimicrobial, anthelmintic and cyclooxygenase-inhibitory activities and phytochemical analysis of *Leucosidea sericea*

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ABSTRACT

Ethnopharmacological relevance: Leucosidea sericea is used as a vermifuge and in the treatment of ophthalmia by various tribes in southern African countries.
Aim of the study: The study aimed at screening leaves and stems of *Leucosidea sericea* for pharmacological activity and validating the plant's traditional use. A general phytochemical screening was also carried out.
Materials and methods: Petroleum ether (PE), dichloromethane (DCM), ethanol (EtOH) and water extracts of the plant parts were investigated for antimicrobial, anthelmintic and cyclooxygenase (COX) inhibitory activities. Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) and *Candida albicans* were used for the antimicrobial evaluation. *Caenorhabditis elegans* was used for the anthelmintic assay using the microdilution technique. Cyclooxygenase-1 and -2 (COX-1 and -2) were used to evaluate the anti-inflammatory potential of the plant extracts. Phytochemical analysis for phenolic compounds, including gallotannins, condensed tannins and flavonoids was done using 50% methanol extracts of the leaves and stems employing spectrophotometric methods.
Results: The leaf extracts exhibited broad spectrum antibacterial activity ranging from 0.025 to 6.25 mg/ml. The most noteworthy minimum inhibitory concentration (MIC) of 0.025 mg/ml was exhibited by PE and DCM leaf extracts against *Bacillus subtilis* and *Staphylococcus aureus*, respectively. In the anthelmintic assay, the best minimum lethal concentration (MLC) value of 0.26 mg/ml was observed for the DCM and EtOH leaf extracts. Both leaf and stem organic solvent extracts exhibited high to moderate inhibition against COX-1 and -2 at a screening concentration of 250 μg/ml. At lower concentrations, the extracts displayed a dose-dependent inhibition, with the lowest IC_{50} values of 0.06 μg/ml (COX-1) and 12.66 μg/ml (COX-2) exhibited by the PE extract of the leaves. Generally, the leaf extracts exhibited better pharmacological activities and contained higher amounts of phenolic compounds than the stem extracts. Alkaloids and saponins were only detected in the leaf and stem extracts, respectively.
Conclusion: The reported results support the local use of *Leucosidea sericea* against eye infections and as a vermifuge. The pharmacological activities exhibited by the leaf extracts are probably due to their higher phenolic levels.

1. Introduction

Leucosidea sericea Eckl. & Zeyh. belongs to the family Rosaceae and the only species in the genus *Leucosidea*. The plant is native to the Afromontane regions of southern African countries such as South Africa, Lesotho and Zimbabwe. *Leucosidea sericea* occurs at high altitude, often near water, in grassland and on mountain slopes, often growing as a straggling silvery shrub or a dense, small, evergreen tree, 2–9 m in height (Pooley, 1993). It is used to expel parasitic intestinal worms (vermifuge) and as an astringent in combination with other plants. Ground leaf paste is also used for conjunctivitis (ophthalmia) by Zulu traditional healers (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996). However, despite the frequent use of this plant, pharmacological and phytochemical information is limited.

In most developing and less developed countries, helminth infections are a major health concern because they predispose humans to other infections such as fungal and bacterial infections (Cox, 2001). This can lead to incapacitating diseases that inflict severe disability and possibly even death, especially among impoverished people because factors such as poor sanitation, poverty and malnutrition abound in these communities (Brooker et al., 2006).

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Ophthalmia is the inflammation of the conjunctiva of the eyes, which at times, is of bacterial origin. *Bacillus subtilis*, *Neisseria gonorrhoeae* and *Staphylococcus aureus* have also been implicated in ophthalmia caused by bacteria (Murray et al., 1998). Inflammatory responses could be linked to these ailments as inflammation is one of the major contributing factors to the exacerbation of many disease states (Iwalewa et al., 2007; Carretero et al., 2008).

As in the case of most developing countries, many South Africans depend on traditional medicine because of its presumed affordability, availability and cultural importance (Mander, 1998). Plant materials and extracts are mainly prescribed by traditional healers for the treatment of various diseases. Due to the absence of standard investigating procedures for evaluating the cause of diseases, common symptoms observed in patients determine the type of plant used (Hewson, 1998). The variety of chemicals often present in a plant extract have different pharmacological activities such as anthelmintic, antimicrobial and anti-inflammatory, which may act in a synergistic manner resulting in the overall clinical effect (Gurib-Fakim, 2006). Therefore, the use of multiple bioassays in pharmacological testing is important as it gives a clearer indication of the effect of the extracts in relation to the disease state (Houghton et al., 2007). In addition, it reduces the possibility of losing other potentially useful bioactive compounds present in the investigated plant extracts (Rates, 2001; Houghton et al., 2007). For the above reasons, *Leucosidea sericea* extracts were screened for antimicrobial and anthelmintic, as well as cyclooxygenase (COX) inhibitory activities. A preliminary phytochemical analysis was also carried out to determine the presence of different classes of secondary metabolites.

2. Materials and methods

2.1. Plant collection and extract preparation

Plant parts were collected during summer (March 2009) from the University of KwaZulu-Natal (UKZN) Botanical Garden, Pietermaritzburg, South Africa. The plant was identified and a voucher specimen was prepared (A.AREMU 4 NU) and lodged in the Bews Herbarium, UKZN, Pietermaritzburg, South Africa. The leaves and stems were dried at 50 °C, ground into a powder through a 1-mm ring sieve using an Ultra-Centrifugal Mill (ZM 200, Retsch®, Germany) and stored at room temperature in airtight containers under dark conditions.

Ground material (1 g) was extracted independently with 10 ml of petroleum ether (PE), dichloromethane (DCM), ethanol (EtOH) and water in an ultrasonic sonicator (Julabo GMBH, West Germany) containing ice water for 1 h. Extracts were filtered under vacuum through Whatman No. 1 filter paper. Organic solvent extracts were concentrated with a rotary evaporator (Büchi, Germany) at 30 °C, water extracts were freeze-dried. Extracts were stored in the dark at 10 °C until required for analysis.

Phenolic compounds were extracted from dry plant material as described by Makkar (2000). Ground plant material (1 g) was extracted with 10 ml of 50% methanol (MeOH) by sonicating in ice cold water for 20 min before filtering and used for the assay.

2.2. Pharmacological evaluation

2.2.1. Antibacterial assay

Minimum inhibitory concentration (MIC) values of extracts were evaluated using the microdilution assay in a 96-well microtitre plate (Eloff, 1998a). Overnight cultures of two Gram-positive (*Bacillus subtilis* ATCC 6051, 4.8×10^9 CFU/ml and *Staphylococcus aureus* ATCC 12600, 2.7×10^9 CFU/ml) and two Gram-negative (*Escherichia coli* ATCC 11775, 7.0×10^{10} CFU/ml and *Klebsiella pneumoniae* ATCC 13883, 2.5×10^9 CFU/ml) bacteria were diluted with sterile Mueller–Hinton (MH) broth. The final concentration of the bacteria in the assay was approximately 10^6 CFU/ml. Organic solvent extracts were redissolved in DMSO, and water extracts in sterile water to make 50 mg/ml. This resulted in a final extract concentration that ranged from 12.5 to 0.01 mg/ml in the wells of the microtitre plate. Neomycin (100 μl; 0.4 mg/ml) (Sigma–Aldrich, Germany) was used as a reference drug against each bacterium. MH broth, DMSO and water were included as controls. Bacterial growth was indicated by adding 50 μl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) (Sigma–Aldrich). MIC values were recorded as the concentrations of the lowest clear wells of each extract. The assay was repeated twice in duplicate per extract.

2.2.2. Antifungal assay

The antifungal activity of the extracts was evaluated against *Candida albicans* (ATCC 10231, $5 \times l0^5$ CFU/ml) using the microdilution assay (Eloff, 1998a) with slight modifications (Masoko et al., 2007). The final concentration of *Candida albicans* in the assay was approximately 10^6 CFU/ml while the extract concentrations ranged from 12.5 to 0.01 mg/ml. Amphotericin B (100 μl; 0.25 mg/ml) (Sigma–Aldrich, Germany) was used as a reference drug. *Candida albicans* culture, yeast malt (YM) broth, DMSO and water were included as controls in the assay. Fungal growth was indicated by adding 50 μl of 0.2 mg/ml INT. MIC values were recorded as the concentrations of the lowest clear wells of each extract. Fungicidal activity was determined by adding YM broth (50 μl) to all clear wells with further incubation for 24 h. Minimum fungicidal concentration (MFC) values were recorded as the concentrations of the lowest clear wells of each extract. The assay was repeated twice in duplicate per extract.

2.2.3. Anthelmintic assay

An *in vitro* colourimetric assay for the determination of free-living nematode larvae viability, as described by James and Davey (2007) with modifications, was used in evaluating the minimum lethal concentration (MLC) values of the extracts against *Caenorhabditis elegans* var. Bristol (N2). *Caenorhabditis elegans* cultures (3-day-old) were prepared by subculturing a stock culture seeded with autoclaved *Escherichia coli* to serve as a non-living food source for the nematodes. The *Caenorhabditis elegans* culture plate was washed with 5 ml of M9 buffer (Brenner, 1974) into a sterile McCartney bottle and the optical density (OD) at 530 nm was measured using a UV–vis spectrophotometer (Varian Cary 50, Australia). Thereafter, 5 ml of M9 buffer was adjusted with an appropriate volume of the prepared *Caenorhabditis elegans* culture to obtain a mixture in the OD_{530} range of 0.04–0.06 (sufficient culture for one microtitre plate). Organic solvent extracts were redissolved in DMSO (25 mg/ml), and water extracts in sterile water (50 mg/ml). Each redissolved extract (100 μl) was two-fold serially diluted with 100 μl sterile distilled water along a 96-well microtitre plate. A similar two-fold serial dilution of 100 μl of 1 mg/ml levamisole (Sigma–Aldrich, Germany) was used as a reference drug. The prepared *Caenorhabditis elegans* culture (50 μl) containing approximately 100 worms were added to each well of the microtitre plate. The final concentration ranged from 8.33 to 0.06 mg/ml and 16.67 to 0.13 mg/ml for organic solvent and water extracts, respectively. *Caenorhabditis elegans* culture, DMSO and water were included as controls in the assay. The microtitre plates were covered with parafilm and incubated at 20 °C for 48 h. Thereafter, 50 μl INT (1.25 mg/ml) was added to the wells of the microtitre plate and further incubated at 20 °C for 24 h. Active organisms biologically reduce the colourless INT to a pink-red colour (McGaw et al., 2007). The concentration of the lowest clear well was recorded as the MLC value of the extract. Extracts were tested in duplicate and the assay was repeated twice.

Table 1
Antimicrobial activity of *Leucosidea sericea* extracts expressed as MIC (mg/ml) against the bacteria and MIC (mg/ml) and MFC (mg/ml) against *Candida albicans*. Values in bold were considered as noteworthy antimicrobial activity.

Plant part	Extract	Antibacterial activity MIC (mg/ml)				Antifungal activity	
		Bacteria				*Candida albicans*	
		B.s.	*S.a.*	*E.c.*	*K.p.*	MIC (mg/ml)	MFC (mg/ml)
Leaves	PE	**0.025**	**0.098**	**0.39**	**0.39**	1.56	1.56
	DCM	**0.098**	**0.025**	**0.39**	**0.39**	3.13	3.13
	EtOH	**0.098**	**0.195**	**0.39**	**0.39**	6.25	6.25
	Water	**0.78**	1.56	3.13	3.13	12.50	>12.50
Stems	PE	**0.39**	**0.39**	1.56	**0.78**	3.13	6.25
	DCM	**0.78**	**0.78**	1.56	1.56	6.25	6.25
	EtOH	**0.78**	**0.78**	1.56	1.56	6.25	6.25
	Water	3.13	3.13	6.25	6.25	>12.50	>12.50
Neomycin (μg/ml)[a]	1.56	1.56	0.39	0.78	–	–	
Amphotericin B (μg/ml)[b]	–	–	–	–	–	0.15	9.80

MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration. *B.s.*, *Bacillus subtilis*; *S.a.*, *Staphyloccocus aureus*; *E.c.*, *Escherichia coli*; *K.p.*, *Klebsiella pneumoniae*. PE, petroleum ether; DCM, dichloromethane; EtOH, ethanol.
[a] Positive control for the antibacterial assay.
[b] Positive control for the antifungal assay.

2.2.4. Cyclooxygenase (COX-1 and -2) inhibition assays

Cyclooxygenase enzymes (COX-1 and -2) assays as described by Jäger et al. (1996) with modifications (Zschocke and Van Staden, 2000) were used to determine the anti-inflammatory potential of the plant extracts. The controls were a solvent blank (EtOH), and a background correction reaction in which the enzymes were inactivated with HCl, and kept on ice before adding [^{14}C] arachidonic acid (16 Ci/mol; 30 μM). Indomethacin (Sigma–Aldrich, Germany) was used as a reference anti-inflammatory drug (5 μM for COX-1 and 200 μM for COX-2). Organic solvent and water extracts were initially tested at 250 μg/ml and 2 mg/ml, respectively. Organic solvent extracts were further tested at three lower concentrations (125, 62.5 and 31.3 μg/ml) while water extracts that had high COX inhibitory activity were also tested at 1 mg/ml. COX inhibition by the extracts was calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank using the equation below:

$$\text{COX inhibition}\,(\%) = \left\{1 - \left(\frac{\text{DPM}_{\text{extract}} - \text{DPM}_{\text{background}}}{\text{DPM}_{\text{solvent blank}} - \text{DPM}_{\text{background}}}\right)\right\} \times 100$$

where DPM is the disintegrations per min of the extract, background and solvent blank. Results are reported as means of two experiments in duplicate and values are expressed as percentage mean ± standard error.

2.3. Phytochemical analysis

The Folin-Ciocalteu (Folin-C) assay using gallic acid as standard was used for the evaluation of total phenolics from the 50% MeOH extracts (Makkar, 2000). Total phenolic concentrations were expressed as gallic acid equivalents (GAE) per gram dry matter.

Gallotannin content was determined using the rhodanine assay as described by Makkar (2000) and its concentrations in the extracts were expressed as GAE per gram dry matter.

Condensed tannin content was evaluated using the butanol–HCl assay as described by Makkar (2000) with slight modifications by Ndhlala et al. (2007) and the percentage dry matter was calculated as equivalent amount of leucocyanidin (LCE) using the equation below:

$$\text{Condensed tannin}\,(\%) = \left(\frac{A_{550} \times 78.26 \times \text{dilution factor of extract}}{\%\,\text{dry matter}}\right) \times 100$$

where A_{550} = absorbance of sample at 550 nm. The formula assumes the effective $E^{1\%,\,1\,\text{cm},\,550\,\text{nm}}$ of leucocyanidin to be 460.

The flavonoid content was evaluated as described by Hagerman (2002), with modifications. In triplicate, 50 μl of each 50% MeOH extract were diluted with 950 μl glacial acetic acid, followed by the addition of 2.5 ml of 4% HCl in methanol (v/v) and 2.5 ml vanillin reagent (4% vanillin in glacial acetic acid, w/v), after which the reaction mixture was incubated for 20 min at room temperature. After incubation, absorbance at 500 nm was measured using a UV–vis spectrophotometer against water blank. Flavonoid content was expressed as catechin equivalents (CTE) per gram dry matter.

To determine the presence of saponins, the froth test for saponins as described by Tadhani and Subhash (2006) was used. The presence of alkaloids was done based on the quantitative method of Makkar and Goodchild (1996). In addition, Mayer's and Wagner's reagents were used to test for the presence of alkaloids in the extracts.

2.4. Statistical analysis

Percentage inhibition values were log-transformed before being subjected to statistical analysis. Data were subjected to one-way analysis of variance (ANOVA) using SPSS software package for Windows (SPSS®, version 10.0 Chicago, USA). Where there was statistical significance ($P \leq 0.05$), the means were further separated using Duncan's multiple range test. The IC_{50} values for COX assays were calculated from the logarithmic non-linear regression curve derived from the plotted data using GraphPad Prism software package for Windows (GraphPad version 4.03, San Diego, USA).

3. Results

3.1. Antimicrobial activity

The MIC values of *Leucosidea sericea* extracts against the test bacteria, as well as the MIC and MFC values against *Candida albicans*, are presented in Table 1. Extracts with MIC or MFC values less than 1.0 mg/ml were considered as having good antimicrobial activity (Aligiannis et al., 2001). All the organic solvent extracts of the leaves showed broad spectrum antibacterial activity, with MIC values ranging from 0.025 to 0.39 mg/ml. Besides the water extract of the leaves, which had an MIC value of 0.78 mg/ml against *Bacillus subtilis*, all the water extracts showed low antimicrobial activity (MIC > 1.0 mg/ml). With the exception of the PE extract

Table 2
Anthelmintic activity of *Leucosidea sericea* extracts expressed as MLC (mg/ml) against *Caenorhabditis elegans*. Values in bold were considered as high anthelmintic activity.

Extract	MLC (mg/ml)	
	Leaves	Stems
Petroleum ether	**0.52**	2.08
Dichloromethane	**0.26**	4.17
Ethanol	**0.26**	2.08
Water	8.33	8.33

MLC, minimum lethal concentration; MLC value of levamisole against *Caenorhabditis elegans* was 40 μg/ml.

Table 3
Inhibitory concentrations at 50% (IC_{50}) of cyclooxygenase enzymes (COX-1 and -2) for *Leucosidea sericea* extracts. Values represent the means ± standard error ($n = 4$).

	Extract	IC_{50} (μg/ml)	
		Leaves	Stems
COX-1	Petroleum ether	0.06 ± 0.02	3.80 ± 0.96
	Dichloromethane	0.13 ± 0.07	24.40 ± 0.84
	Ethanol	7.29 ± 1.52	10.33 ± 3.90
COX-2	Petroleum ether	12.66 ± 2.36	43.03 ± 2.90
	Dichloromethane	21.75 ± 3.65	91.25 ± 2.31
	Ethanol	142.00 ± 3.23	171.27 ± 3.00

of the stems that inhibited *Klebsiella pneumoniae* (Gram-negative bacterium), all organic solvent extracts of the stems only showed noteworthy antibacterial activity against the Gram-positive bacteria. As indicated in Table 1, the extracts exhibited low antifungal activity against *Candida albicans*.

3.2. Anthelmintic activity

The MLC values of the plant extracts against *Caenorhabditis elegans* are presented in Table 2. In this study, MLC values lower than 1 mg/ml, from 1 to 4 mg/ml and above 4 mg/ml were defined as high, moderate and low anthelmintic activity, respectively. All the organic solvent extracts of the leaves showed high anthelmintic activity. All stem extracts, as well as the water extract of the leaves, exhibited moderate to low anthelmintic activity.

3.3. Cyclooxygenase (COX-1 and -2) inhibitory activity

The inhibitory effects of the evaluated plant extracts against COX enzymes are presented in Fig. 1. Plant extracts with a percentage inhibition above 70%, from 40% to 70%, and below 40% were considered as showing high, moderate or low inhibitory activity, respectively (Tunón et al., 1995). At the highest screening concentration (250 μg/ml), the PE extract of the leaves exhibited the highest COX-1 and COX-2 inhibition of 97% and 91%, respectively. Furthermore, with respect to plant parts, leaf extracts showed better COX inhibition than the stem extracts. Low inhibitory activity was observed with water extracts at the highest screening concentration (2 mg/ml), while no activity was observed at a lower concentration (1 mg/ml) (results not shown). Generally, the plant extracts showed better inhibition against COX-1 than COX-2. The calculated IC_{50} values of the organic solvent extracts of both parts are presented in Table 3. The lowest IC_{50} value (0.06 μg/ml) was exhibited by the PE extract of the leaves against COX-1 and the highest IC_{50} value (171.27 μg/ml) was shown by the EtOH extract of the stems against COX-2 (Table 3).

3.4. Phytochemical content

The total phenolics, gallotannin, condensed tannin and flavonoid content in the leaf and stem 50% MeOH extracts are pre-

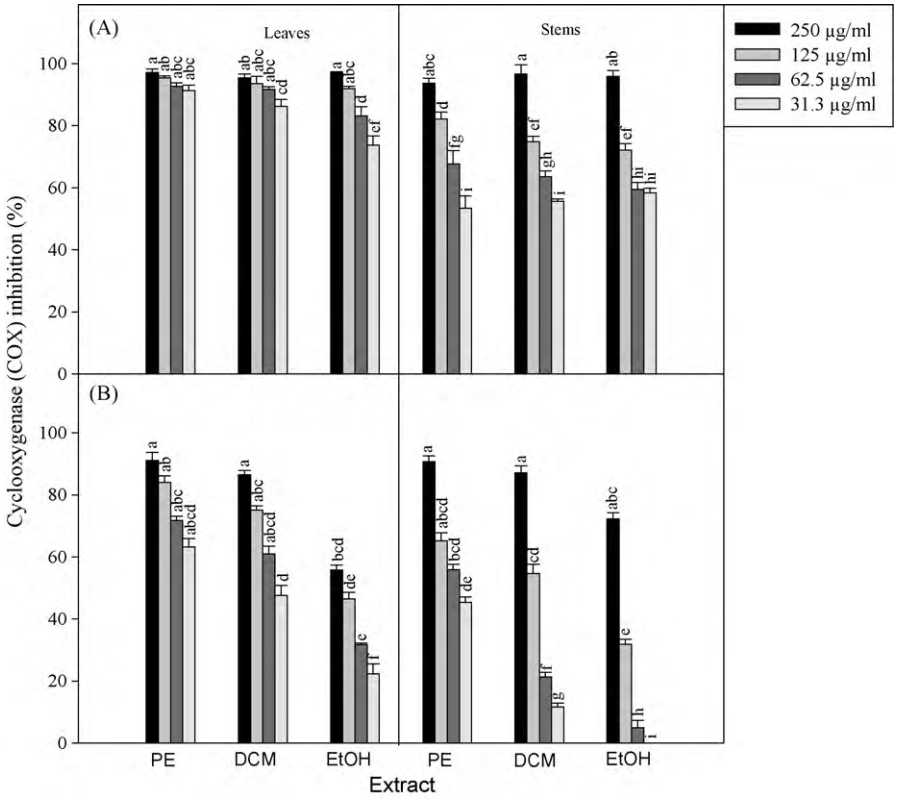

Fig. 1. COX-1 and -2 inhibition (%) by *Leucosidea sericea* extracts at four concentrations (A) COX-1; (B) COX-2 inhibition. COX-1 and -2 inhibition (%) by indomethacin was 78.50 ± 3.38 and 65.10 ± 3.54, respectively. Bars in each graph followed by different letter(s) are significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Table 4
Total phenolics, gallotannin, condensed tannin and flavonoid content of *Leucosidea sericea* extracts. Values represent the means ± standard error ($n = 3$).

Plant secondary metabolites	Leaves	Stems
Total phenolics (mg GAE/g dry matter)	36.66 ± 0.88	6.40 ± 0.24
Gallotannin (μg GAE/g dry matter)	29.32 ± 0.07	5.12 ± 0.20
Condensed tannin (% LCE/g dry matter)	0.46 ± 0.02	0.47 ± 0.20
Flavonoid (mg CTE/g dry matter)	0.66 ± 0.04	0.26 ± 0.02

GAE, gallic acid equivalents; LCE, leucocyanidin equivalents; CTE, catechin equivalents.

sented in Table 4. With the exception of condensed tannin content, higher levels of phytochemicals were contained in the leaves. In addition, the 50% MeOH extract of the leaves indicated the presence of alkaloids and only the stem extract tested positive for saponins.

4. Discussion

Leucosidea sericea showed promising pharmacological potential in this study. The only previous study on the pharmacology of *Leucosidea sericea* reported the presence of aspidinol, an anthelmintic compound with activity against Gram-positive bacteria and *Candida albicans* using the disk diffusion method (Bosman et al., 2004). In this study, other pharmacological activities such as anthelmintic and COX inhibitory activity as well as antibacterial activity against both Gram-negative and Gram-positive bacteria were determined. However, the extracts showed a higher percentage inhibition against COX-1 than COX-2. Although it is ideal to test plant extracts against specific target microorganisms, taxonomical representative species were used in this preliminary antimicrobial screening to avoid handling numerous pathogenic microorganisms. The broad spectrum antibacterial activity exhibited by the leaf extract could be linked to its use for eye infections of bacterial origin in traditional medicine. The anthelmintic activity displayed by the plant extracts could also validate the use of the plant as an anthelmintic in traditional medicine. No noteworthy antifungal activity was observed as all the tested extracts had MIC above 1 mg/ml. Traditional healers usually prepare aqueous or alcohol extracts of medicinal plants. The use of water for plant material extraction naturally limits the type and amount of compounds extracted from plant material (Eloff, 1998b). Although poor pharmacological activities were exhibited by the tested water extracts, the extraction of the medicinal plant material with solvents of varying polarity in this study was relevant for possible isolation of their bioactive principle(s). The COX inhibition exhibited by the PE extract of the leaves make *Leucosidea sericea* a promising candidate for further work in the search for novel anti-inflammatory compounds.

Phenolic compounds including gallotannins, condensed tannins and flavonoids as well as saponins and alkaloids have been implicated in pharmacological activities such as anthelmintic, antimicrobial and anti-inflammatory activities (Makkar et al., 2007). There is no literature information on the phytochemical composition of *Leucosidea sericea* (Bosman et al., 2004). The finding of alkaloids in *Leucosidea sericea* leaf extracts emphasizes its pharmacological potential. The astringent property of *Leucosidea sericea* as a result of the quantity of tannins could be the reason for the use of the plant as anthelmintics in traditional medicine. All of the pharmacological activities exhibited by *Leucosidea sericea* leaf extracts raise some interesting expectations, indicating that further studies on the biological properties and the isolation of active components in the plant part should be performed.

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