

## Original study

## ***In-vitro* antibacterial sensitivity of *Usnea barbata* lichen extracted with methanol and ethyl-acetate against selected *Staphylococcus* species from milk of cows with mastitis**

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### **Abstract**

This study aimed at evaluating the antimicrobial potential of *Usnea barbata* lichen as a medicinal plant against selected *Staphylococcus* species isolated from raw milk of cows. *In-vitro* screening of methanol and ethyl-acetate extracts from *Usnea barbata* lichen were evaluated to determine their antimicrobial activity against thirteen different *Staphylococcus* species. The selected organisms were isolated from raw bovine milk and identified using several biochemical tests and confirmed with API staph kit. The antimicrobial activity of the extracts were evaluated using both the agar well diffusion method (at 5 mg/ml, 10 mg/ml and 20 mg/ml) and the broth micro-dilution technique to determine the mean zone of inhibition and the minimum inhibitory concentration (MIC), respectively. Both the methanol and ethyl-acetate extracts showed variable antimicrobial activity against the *Staphylococcus* species with mean zones of inhibition ranging from 0-34 mm in diameter at 5 mg/ml, 10 mg/ml and 20 mg/ml, respectively. Susceptibility by the *Staphylococcus* species tested in the methanol and the ethyl-acetate extract was 92.31 % and 53.85 %, respectively. The MIC result for the methanol extract ranged from 0.04 to 10 mg/ml, while that of the ethyl-acetate extract ranged from 0.16 to 5 mg/ml. Results from this study revealed the *in vitro* microbial activity of *Usnea barbata* extracts which indicate its potential as a medicinal plant.

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**Keywords:** antimicrobial resistance; mastitis; microbial activity; medicinal plant

**Abbreviations:** DMSO: dimethyl sulfoxide; MIC: minimum inhibitory concentration

## Introduction

Mastitis, an inflammation of the mammary gland especially in dairy animals is known to be a huge threat due to the resistance of several causative organisms to antimicrobial agents, leading to considerable economic losses in farms (Halasa *et al.* 2009). Mastitis in cows could be clinical or subclinical in nature. Clinical mastitis can be visibly seen from the appearance of raw milk of cows but in subclinical mastitis there is no apparent change in milk (Giannechini *et al.* 2002). *Staphylococcus* species are among the several pathogenic organisms that cause mastitis and are known to be resistant to a wide range of antimicrobial agents in several parts of the world (Pyorala & Taponen 2009, Sampimon *et al.* 2011). In South Africa, a study conducted by Petzer *et al.* (2009) indicated that *Staphylococcus* species are the most prevalent pathogens (with increasing trend on yearly bases) that cause mastitis in dairy cows.

Antimicrobials are widely used for treatment of various ailments both in humans and animals. Over the years, the continuous use of antimicrobials in the dairy sector has led to the emergence of resistant strains of several pathogens that are linked to the cause of mastitis disease in animals (Pitkala *et al.* 2004). Mastitis disease causing organisms develop resistance to antimicrobials through a systematic process which make them able to withstand the effect of drugs used against them (Suleiman *et al.* 2010). Even though several efforts have been made to combat the emergence of these organisms, by way of introducing new antimicrobial agents there is yet no lasting solution to the problem at present (Gould 2009). The global challenge of antimicrobial resistance to drugs is bothersome. Thus, the search for other effective drugs to address the situation would be worthwhile (Dhanalakshmi *et al.* 2013).

Medicinal plants have shown potential and some have been discovered to possess active antimicrobial ingredients both in humans and animals (Alviano & Alviano 2009, Madamombe & Afolayan 2003). Several bacterial resistant organisms are reported to surpass the effect of newly developed antibiotic drugs (Gould, 2009). The rise in the trend of resistance in mastitis causing organisms to antibiotics is posing a serious challenge to the society (Tiwari *et al.* 2013).

Many small-scale farmers have resorted to the use of herbal plants as alternatives to treat their animals (Masika *et al.* 2002). This practice has increased greatly in Africa. According to Ndip *et al.* (2007), Africa has the highest record of herbal plants usage as options for treating various diseases both in humans and in animals.

*Usnea barbata* lichens are epiphytes that grow on leaves of other trees and plants in a symbiotic relationship. Like other lichens they can also grow on rocks and soils with very low nutrient content (Vrablikova *et al.* 2006). The use of *Usnea barbata* for the treatment of mastitis in cattle by rural dwellers in the Eastern Cape of South Africa was reported by Madamombe & Afolayan (2003). According to Madamombe & Afolayan (2003), several rural farmers use the decoction prepared from the lichen to treat cattle orally against mastitis disease.

Presently, we are unaware of any study that has evaluated the antimicrobial activity of *Usnea barbata* plant on *Staphylococcus* species isolated from raw milk of cows with mastitis. The current study aimed to evaluate the *in-vitro* antimicrobial activity of methanol and ethyl-

acetate extracts of *Usnea barbata* lichens on some selected *Staphylococcus* species isolated from raw milk of cows.

## Material and methods

### *Isolation and identification of Staphylococcus species*

Milk samples from cows were collected from a commercial dairy farm and kept in a cooler box with ice for bacterial analysis in the laboratory. They were immediately cultured on mannitol salt agar on arrival in the laboratory then kept in an incubator at 37 °C for 24 h. Milk samples that were presumed to be positive for *Staphylococcus* species were sub-cultured in nutrient agar to get pure colonies of species isolates. Several other biochemical tests including Gram staining, catalase and oxidase test were also carried out. Isolates were finally identified as *Staphylococcus* species (to their species level) using API staph kit (bioMerieux, Marcy l' Etoile, France).

### *Plant sample and extracts preparation*

*Usnea barbata* was harvested from the Hogsback forest (32° 34' 60 S; 26° 56' 60 E) about 30 km from Alice town, Eastern Cape, South Africa. The *Usnea barbata* lichen was selected based on the information of their use for the treatment of cattle with mastitis by farmers in that area. Identification of the plant was done at the Department of Botany, University of Fort Hare where voucher specimens (Idah 2000/2) have been deposited.

The *Usnea barbata* lichen was air-dried at room temperature (25 °C) for 10 days and thereafter ground into powder before it was serially extracted with methanol and ethyl acetate solvent, respectively. Extraction was done using a portion of 400 g of the *Usnea barbata* lichen in an extraction bottle before adding the solvents (methanol and ethyl-acetate) and then shaken for 24 h in a shaker (Edison, N.J., USA). After 24 h, the mixture was centrifuged at 1 500 rpm for 10 min and filtered using a Whatman No. 1 filter paper. Filtrate was concentrated to dryness under reduced pressure at 40 °C in a rotary evaporator (Strike 202, Steroglass, Perugia, Italy). Extracts were stored in a tight lid container for further use.

### *Testing for antimicrobial sensitivity*

The agar well diffusion technique was used to test the antimicrobial sensitivity of *Staphylococcus* bacteria to plant extracts. Break point with an inhibition zone of diameter of  $\geq 11$  was chosen for bacterial susceptibility for the plant extracts and the antibiotic (Nyenje & Ndip 2011). Few colonies of bacteria isolates from freshly prepared (within 24 h) nutrient agar were added to sterile normal saline in a test tube with turbidity adjusted to  $1 \times 10^8$  CFU/ml (McFarland number) to make the required inocula for the experiment. A sterile cotton swab was thereafter used to inoculate the standardized bacterial suspension in a radial pattern on Mueller Hinton agar plates (Oxoid, Basingstoke, England). The plates were afterwards left to dry for about 5-10 min. Holes of about 6 mm in diameter were then aseptically punched to make wells (5 holes per plate) in the Mueller Hinton agar with the aid of a sterile cork borer. Each well was thereafter filled with 50  $\mu$ l of extract at different concentrations (5, 10 and 20 mg/ml). The extracts (methanol and ethyl acetate) were dissolved in 5 % dimethyl sulfoxide (DMSO) before adding them into

the wells. The negative control was done by adding 50 µl of 5 % DMSO into the holes of the Mueller Hinton agar plates containing the inocula. The 5 % DMSO was prepared by adding 5 ml of concentrated solvent (DMSO) into 95 ml distilled water. Each plate was made in triplicate and left for 30 min for sufficient diffusion of the extracts into the agar before they were incubated at 37 °C for 24 h. The zones of inhibition were measured to the nearest millimetre after 24 h. The mean zone of inhibition was calculated for each solvent; 0.01 mg/ml of amoxicillin was used as positive control while DMSO (5 %) was used as negative control.

#### *Determination of minimum inhibitory concentration of plant extracts*

Minimum inhibitory concentration (MIC) of the plant extracts against the bacterial species was determined using the broth micro-dilution method in 96-well micro-titre plates. Series of dilutions were made for the extracts and for the standard antibiotic (amoxicillin) with concentrations ranging from 0.01 to 10 mg/ml in 5 % DMSO. The DMSO solution was dissolved in sterile distilled water to the desired concentration. Two-fold serial dilution of stock solution from extracts (10 mg/ml) together with Mueller Hinton broth was prepared in the micro-titre wells and a standardized bacterial suspension (20 µl) added into the wells except for the control wells, which contained broth and sterile distilled water, respectively. Plates were then incubated for 24 h at 37 °C. A drop of rezasurin solution as an indicator was added to the micro titre wells to serve as an indicator. Bacterial growth was indicated by a colour change from purple to pink. The least concentration in the wells of the test solution that led to inhibition of growth was taken as the MIC.

#### *Statistical analysis*

The SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. One way ANOVA test was used to evaluate if there was any significant difference in the diameter of zones of inhibition of the plant extracts and the standard antibiotic (amoxicillin). Statistical significance was considered at  $P < 0.05$ .

## **Results**

The zones of inhibition for methanol extract ranged from 10 to 34 mm while that of ethyl-acetate ranged from 0 to 23 mm (Table 1). Amoxicillin which was used as a positive control gave a zone of inhibition in the range of 17 to 47 mm (Table 1). The DMSO (5 %) used as negative control showed no activity. With reference to the break point (inhibition zone diameter  $\geq 11$ ), 6 out of the 13 bacterial strains were the most resistant organisms to both methanol and ethyl-acetate extract viz, *S. haemolyticus*, *S. capitis*, *S. cohnii-urealyticus*, *S. cohnii-cohnii*, *S. hominis* and *S. saprophyticus*. On the other hand, four strains: *S. xylosus*, *S. sciuri*, *S. lentus* and *S. epidermidis*, were the most susceptible organisms. There was statistical significance in the mean zone of inhibition of the standard antibiotic (amoxicillin) and the plant extracts (methanol and ethyl-acetate) at  $P < 0.05$  (Table 1).

Out of the 13 *Staphylococcus* species that were tested, the susceptibility of the bacterial organisms to the standard antibiotic (amoxicillin) and methanol extract was 100 % and 92.31 %, respectively while that of the ethyl-acetate extract was 53.85 % (Figure 1). The MIC

results showed that methanol and ethyl-acetate had an antimicrobial activity ranging from 0.04 to 10 mg/ml and 0.16 to 5 mg/ml, respectively while the MIC for amoxicillin on the other hand had a range from 0.63 to 10 µg/ml (Table 2).

Table 1

Zone of inhibition (mm) of *Usnea barbata* extracts and amoxicillin against the test organisms

Species	Solvent extracts of <i>Usnea barbata</i> plant, mg/ml						amoxicillin 0.01
	5	methanol 10	20	5	ethyl-acetate 10	20	
<i>S. aureus</i>	14±1	-	-	0±0	0±0	15±0.58	23±1.50
<i>S. sciuri</i>	15±1	17±2	-	13	14±1.80	15±3	30±0.57
<i>S. xylosus</i>	34±1.70	-	-	23±1.20	-	-	47±1.50
<i>S. chromogenes</i>	17±1	18±0.57	-	13±0.70	15±1.70	-	21±3.10
<i>S. lentus</i>	14±1.70	17±1	19	13±0.57	14±1.70	-	17±0.57
<i>S. cohnii</i> <sup>a</sup>	10±1.50	-	-	12	14±0.90	19±1.50	39±1.50
<i>S. haemolyticus</i>	16±0.60	-	-	8±7.50	9±6.30	14±1.70	27±1.50
<i>S. capitis</i>	16±14	-	-	0	9±6	10±8	30±6.50
<i>S. epidermidis</i>	26±1.50	-	-	16±20	19±1.60	21±0.60	37±2.10
<i>S. warneri</i>	22±30	-	-	14±0.50	15±0.40	16±2	40±0.60
<i>S. cohnii</i> <sup>b</sup>	18±1.70	-	-	0±0	7±5	9±7	36±1.10
<i>S. hominis</i>	22±1.70	-	-	0±0	14±1.40	17±2.50	39±1
<i>S. saprophyticus</i>	14±1	-	-	0±0	10±6	12±10	20±2

<sup>a</sup>*S. cohnii* – *cohnii*, <sup>b</sup>*S. cohnii* – *urealyticus* (\*values are in mean, ±standard deviation, n=3)

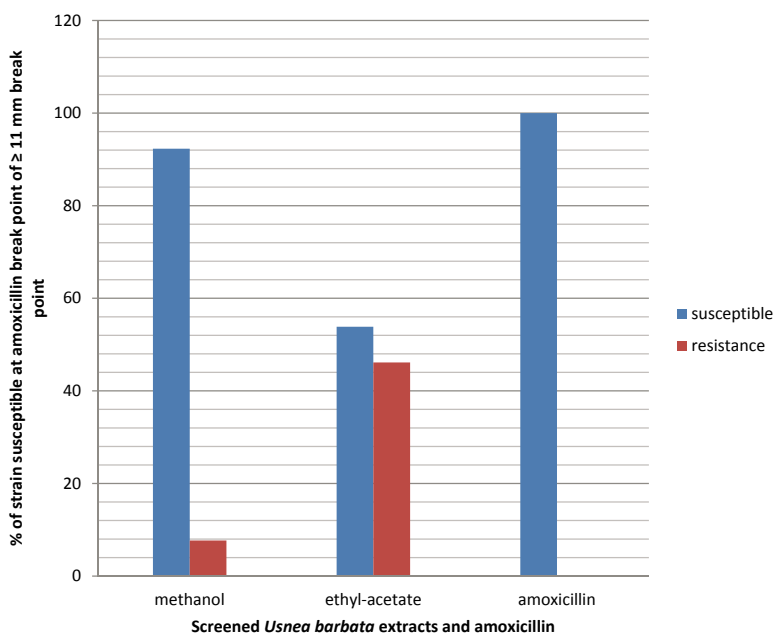


Figure 1

Sensitivity of test organisms to amoxicillin, methanol and ethyl acetate extracts at 5 mg/ml.

Table 2

Antibacterial activity of *Usnea barbata* extracts and amoxicillin against the test organisms

<i>Staphylococcus</i> species	methanol	MIC <sup>a</sup> (mg/ml)	
		ethyl-acetate	amoxicillin
<i>S. aureus</i>	1.25	2.50	6.25±10 <sup>-4</sup>
<i>S. sciuri</i>	3.13±10 <sup>-1</sup>	1.56±10 <sup>-1</sup>	3.13±10 <sup>-4</sup>
<i>S. xylosus</i>	na <sup>b</sup>	na	7.81±10 <sup>-5</sup>
<i>S. chromogene</i>	10 <sup>c</sup>	1.25	6.25±10 <sup>-4</sup>
<i>S. lentus</i>	10	6.25±10 <sup>-1</sup>	5±10 <sup>-3</sup>
<i>S. cohnii-cohnii</i>	3.13±10 <sup>-1</sup>	3.13±10 <sup>-1</sup>	2.50±10 <sup>-3</sup>
<i>S. haemolyticus</i>	6.25±10 <sup>-1</sup>	6.25±10 <sup>-1</sup>	1.25±10 <sup>-3</sup>
<i>S. capitis</i>	6.25±10 <sup>-1</sup>	1.56±10 <sup>-1</sup>	1.56 <sup>-4</sup>
<i>S. epidermidis</i>	1.56±10 <sup>-1</sup>	3.13±10 <sup>-1</sup>	1.56 <sup>-4</sup>
<i>S. warneri</i>	3.90±10 <sup>-2</sup>	3.13±10 <sup>-1</sup>	2.50±10 <sup>-3</sup>
<i>S. cohnii-urealyticus</i>	3.13±10 <sup>-1</sup>	3.13±10 <sup>-1</sup>	6.25±10 <sup>-4</sup>
<i>S. hominis</i>	6.25±10 <sup>-1</sup>	2.50	1.00±10 <sup>-2</sup>
<i>S. saprophyticus</i>	10	5	5±10 <sup>-3</sup>

<sup>a</sup>minimum inhibitory concentration, <sup>b</sup>not active, <sup>c</sup>highest concentration of extract tested

## Discussion

The screening of the antimicrobial properties of *Usnea barbata* lichen in the current study indicated a significant activity with a range between 0 to 34 mm against all the *Staphylococcus* species that were tested. Most papers have reported the antimicrobial activity of *Usnea barbata* on bacteria and fungi isolated from human isolates (Weckesser *et al.* 2007, Kala & Senthilkuma, 2010, Wendakoon *et al.* 2012) but information regarding the use of *Usnea barbata* extracts on bacteria isolates from animal origin (raw milk) is sparse.

In the current study, the range of mean zone of inhibition for all organisms tested was between 7 to 34 mm. The least mean zone of inhibition was observed for *S. cohnii-urealyticus*, *S. haemolyticus* and *S. capitis* while the highest zone of inhibition was observed for *S. xylosus*, *S. sciuri*, *S. lentus* and *S. epidermidis*. In another study by Wendakoon *et al.* (2012), it was observed that the mean zone of inhibition for *S. aureus* and *S. epidermidis* isolates (from human origin) was between 11 to 32 mm; with chemical compounds including flavonoids, tannins, lignins and phenolic acid extracted from the lichen mentioned to be responsible for inhibiting the growth of the bacteria. These chemical compounds are known to initiate antimicrobial activities in many plants because they produce biological effect on microorganisms (Wendakoon *et al.* 2012).

Rankovic *et al.* (2012) in their study identified usnic acid, norstictic acid, atranorin and chloroatranorin from acetone extract of *Usnea barbata* lichen as the active compounds that may be responsible for antimicrobial activity on bacteria. Usnic acid is a chemical component that is extensively produced from extracts of *Usnea* lichens (Cansaran *et al.* 2006) which has been demonstrated to possess potent inhibitory effect against a tumour promoting micro-organism that could lead to cancer (Yamamoto *et al.* 1995) and against *Propionibacterium acnes* that causes acne in humans (Ray *et al.* 2013). Therefore the same compound among others might play a role in inhibiting the growth of the tested organisms.

The differences in the inhibitory activity on the different *Staphylococcus* species in the current study may be due to the structural makeup of the bacterial cells. The thickness of

bacterial cell membranes plays a role in the effectiveness (antimicrobial and bactericidal) of antibiotics (Fennell *et al.* 2004; Okeleye *et al.* 2013). However, the inhibitory activities of the *Usnea barbata* lichen against the tested organisms in the current study indicate that they could be effective for treatment of cattle with mastitis disease caused by the selected *Staphylococcus* species.

There is a dearth of information in literatures about the bacterial effect of ethyl-acetate extracts from *Usnea barbata* lichens. Though, ethyl-acetate has been used as an extraction solvent for other plants including *Peltophorum africanum* and *Combretum molle* (Okeleye *et al.* 2010; Nyenje & Ndip, 2011). Extracts of *Usnea barbata* lichen from other solvents (such as acetone, methanol, carbon dioxide, water) have also been reported to be active against several bacterial organisms including *S. aureus*, *S. epidermidis*, *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli* and *Micrococcus viridans* (Madamombe & Afolayan, 2003; Cansaran *et al.* 2006; Weckesser *et al.* 2007; Rankovic *et al.* 2012; Wendakoon *et al.* 2012). In the current study, a good proportion of the bacterial species was susceptible to both the methanol and the ethyl-acetate extracts. All the bacterial organisms that were screened in the standard antibiotic (amoxicillin) were susceptible while the susceptibility of the tested bacterial organisms to methanol and ethyl-acetate extracts gave 92.31 % and 53.85 % respectively. This may be due to the fact that methanol solvent extracted more microbial inhibiting active compounds from the plant lichen because of its high polarity. In the study conducted by Parekh *et al.* (2005), methanol extract from twelve different plants gave a better antimicrobial activity than water (a lesser polar solvent) when screened against five different bacteria strains including *S. epidermidis*. According to Abu-Shanab *et al.* (2006) and Nyenje & Ndip (2011), organic solvents extract antimicrobial substances from medicinal plants more effectively compared to other solvents.

Several other studies about the activity of *Usnea barbata* lichen against bacterial organisms have also been reported. The MIC value observed by Rankovic *et al.* (2012) in their study ranged between 0.13 and 12.50 mg/ml for ten different bacterial organisms including *S. aureus*, *Bacillus subtilis*, *Escherichia coli* and *Bacillus subtilis* in acetone extract which is close to the result in the current study. Madamombe & Afolayan (2003) and Cansaran *et al.* (2006) also reported a significant antimicrobial activity with MIC value of 0.10 mg/ml for *S. aureus* in methanol and acetone extract. However, the MIC value for the methanol extract against *S. warneri* in the current study was as low as 0.04 mg/ml though the overall activity of both extracts for all the tested organisms varies between 0.04-10 mg/ml. This result suggests that *Usnea barbata* lichen extracted with methanol and ethyl-acetate solvents possess some potential antimicrobial compounds that inhibited the tested organisms. The variation in activity of the selected bacterial organisms screened in the *Usnea barbata* lichen (extract) may not be clearly understood in the current study. Further investigation concerning the isolation, identification and testing of specific compounds of this plant against the tested microbial organisms (using other solvents) may be required in the present search for new antimicrobial drugs.

In conclusion, the current study showed that methanol and ethyl-acetate extracts of *Usnea barbata* exhibited *in-vitro* antimicrobial activities with the methanol extracts being more active. The results of the antimicrobial activity of *Usnea barbata* in the current study also suggest the rationale behind the traditional use of *Usnea barbata* lichen for the treatment of



mastitis in cattle by local farmers in the Eastern Cape Province of South Africa. These lichens are not part of the endangered species identified by the South African government. They are widely spread in several parts of South Africa, including Hogsback forest and in the western region of Port Elizabeth, both areas are located in the Eastern Cape Province of South Africa thus, makes it practicable to isolate active compounds from the plant for a broader usage. It is therefore proposed that further investigation should be carried out on the plant lichen to determine the natural bioactive compounds present in the plant.

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