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Potential Use of *Schkuhria Pinnata* in the Control of Mastitis Pathogens

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Abstract:
This study investigated the potential use of Dwarf Marigold (Schkuhria pinnata) in the control of mastitis pathogens. Schkuhria pinnata (SP) juice extracted using either distilled water or methanol as diluents with selected concentrations ((20%, 40%, 80% and 100%) was tested for antibacterial activity against Staphylococcus aureas, Streptoccus agalactiae and Escherichia coli bacteria. Total Bacterial Count (TBC) for each extract concentration was then determined by visual assessment through plate count on bacterial colonies. Although plant extracts were not as effective as Gentamycin (commercial control), SP extracts had a significant antibacterial activity ($P<0.05$) against Staphylococcus aureas, Streptococcus agalactiae and Escherichia coli bacteria. Type of diluent had no effect ($P>0.05$) on bacterial counts. Escherichia coli had significantly lower ($P<0.05$) TBC than Staphylococcus aureas and Streptococcus agalactiae. Schkuriah pinnata extracts could be considered as potentially effective antibacterial agent and can be developed as an alternative therapy against bovine mastitis.

Keywords: *antibacterial activity, mastitis, plant extracts,*

1. Introduction

The dairy industry in Zimbabwe has been declining since the last decade (Mupunga and Dube, 2012). Milk and dairy products are becoming expensive as high production costs are passed on to the consumer (Marecha, 2009). Prior to Fast Track Land Reform Programme of 2000, the dairy industry comprised of 375 large-scale producers and the number has since declined to 223, while annual milk volumes have declined from 256 million litres to 70 million litres (Marecha, 2013).

This reduction in milk production has been due to poor genetics, poor feed quality, high costs of purchased feed, poor skills and knowledge of good dairy practice (Raza, 2012). Adding to this, are aggregate losses and major deductions from economic production as a result of mastitis disease (Katsande *et al.*, 2010; Marecha, 2009). More than 15% of cows worldwide are rejected each year because of mastitis (Raza, 2012). A recent study carried out in Zimbabwe has shown that there is an approximately 30% prevalence of mastitis especially among resource poor dairy farmers (Katsande *et al*., 2010*)* and about 40% decline in milk production is due to the disease (Marecha, 2009). The prevalence of mastitis is significantly higher in dairy animals than in traditional animals among African countries such as Tanzania (Missanjo, 2010).

Mastitis occurs when white blood cells (leucocytes), are released into the mammary gland in response to an invasion of the teat canal by pathogens such as bacteria (Bailey and Jones, 2010). There are twelve known bacteria that cause mastitis, but Staphylococcus spp, Escherichia coli and Klebsiella spp. are the most common bacterial isolates (Katsande et al., 2010). The bacteria usually damage milk secreting tissue and mammary gland leading to chemical, mechanical, or thermal injury (Bailey and Jones, 2010). The end result is decreasedmilk quantity as well as decreased casein - contributing to a decrease in calcium as most calcium in milk is associated with casein (Hogeveen et al., 2011).

In Zimbabwe, mastitis can be controlled using long acting antibiotics such as Vancomycin, Gentamycin, Neomycin and Penicillin among others (Myllys and Rautalla, 2005). The aforesaid conventional drugs are expensive and usually are out-of-reach for resource

poor smallholder farmers in Zimbabwe (Mwale et al., 2005). There are also limitations to the use of antibiotics as they only reduce the severity of the condition but do not prevent new infection (Marecha, 2009). Moreover, some traces of Gentamycin elements have been reportedly found in milk, thereby raising concerns on consumers' health (Nickerson, 2000). According to Vikki (2002) mastitis is therefore the most 'expensive' disease to dairy farmers as it leads to the highest antibiotic use in dairy production.

Schkuhria pinnata is commonly known as Dwarf Marigold or ruhwahwa (in Shona), and it is widespread as a weed of arable and disturbed ground like fields and roadsides in Zimbabwe (GRIN, 2012). The plant has been used in treating various disorders in animals particularly when the disease was linked to infectious microorganisms (McGaw et al., 2008). In South Africa it has been used to treat eye infections, pneumonia, heart water and diarrhoea in cattle (Merwe et al., 2001), treatment of wounds and retained placenta in livestock (Luseba et al., 2007). On the other hand, the herb has also been used as human medicine to treat chest pains and liver pains in Southern and Eastern Africa (Kokwaro, 1993; Muthaura et al., 2009; Molebatsi et al., 2010). There is therefore need to find alternatives such as Schkuhria pinnata plant which has traditionally been used to treat human and livestock health problems. However no research has been done on the effectiveness and dosages of Schkuhria pinnata required in treating mastitis. The purpose of this study was to determine the potential use of Schkuhria pinnata in the control of mastitis pathogens through its effects on total bacterial counts.

2. Materials and Methods

2.1. Study Site

The research was carried out at the Central Veterinary Laboratories of the Ministry of Agriculture, Mechanization and Irrigation Development (Department of Veterinary Services), in Harare, Zimbabwe.

2.2. Source of Plant Material

Schkuhria pinnata plant material was collected from Ruzawi farming area. Ruzawi is located on latitude 18° 27' South and longitude 31° 34' East, at an altitude of 1369 m above mean sea level (MSL) 10 km south of Marondera town.

2.3. Plant Identification

The plant was positively identified at the National Herbarium and Botanic Garden. Samples containing fresh flowers, leaves, stem and roots from plants were washed several times using running tap water, rinsed thoroughly with sterilised distilled water and then allowed to dry.

2.4. Test Microorganisms

The microbial strains which include Staphylococcus aureas, Streptococcus agalactiae and Escherichia coli were obtained from the Bacteriological Department at the Central Veterinary Laboratories.

2.5. Plate Preparation

The pour plate method was used for the experiments whereby 1 µml of bacterial solution was placed in a petri-dish using a sterile pipette. Different Schkuhria pinnata levels including the controls would then be introduced to the bacteria in the petri-dishes. Blood agar was used as growth media for Streptococcus agalactiae, Staphylococcus aureus and Escherichia coli. Reaction time of 30 minutes was allowed for all samples, while incubation was done for 48 hours at 37˚C.

2.6. Plant Extract Preparation and Experimental Design

A 5 × 2 × 2 × 3 factorial experiment with five treatments, two levels of autoclaving, two types of diluents and three bacterial species replicated four times were used as shown in Table 1. Two different solvents were used in extraction: distilled water and methanol. 20g of cleaned fresh leaves were weighed using Mettler-PJ3000 analytical balance into one litre beakers. Schkuhria pinnata entire fresh plant was crushed using a pestle and mortar and 200 ml of solvent added to the slurry. All solutions (distilled water and Schkuhria pinnata juice extract) were left at room temperature for 24 hours. The plant residues were filtered using Whatman filter paper number one. The extracts were evaporated in a beaker using a Bunsen burner. Half the extract quantities were sterilised using an autoclave for 15 minutes at 140ºC. A serial dilution of Schkuhria pinnata extracts at different graded levels (20%, 40%, 80% and 100%) were made with water.

Treatments	Concentration of *Schkuhria pinnata* extract	Sterilisation	Diluents	Bacterial species	Replicates
1	20	Autoclaved	Methanol	3	4
			Distilled water	3	4
		Non-autoclaved	Methanol	3	4
			Distilled water	3	4
2	40	Autoclaved	Methanol	3	4
			Distilled water	3	4
		Non-autoclaved	Methanol	3	4
			Distilled water	3	4
3	80	Autoclaved	Methanol	3	4
			Distilled water	3	4
		Non-autoclaved	Methanol	3	4
			Distilled water	3	4
4	100	Autoclaved	Methanol	3	4
			Distilled water	3	4
		Non-autoclaved	Methanol	3	4
			Distilled water	3	4
5	Control (Gentamycin)	As per supplier instruction		3	4

Table 1: Experimental design and treatments

2.7. Data Collection

Samples were removed from the incubator after 48 hours and plate count on bacterial colonies were done by visual assessment. Total Bacterial Count (TBC) calculations were done using the following formula:

TBC=Number of colonies observed x Inverse value of dilution factor

2.8. Data analysis

Data was analysed using the Statistical Analysis System (SAS) Version 9.3 (SAS, 2004). Data was tested for normality using the Proc Univariate procedure after which it was ranked using the Proc Ranks procedure and the ranks were analysed using the Proc GLM of SAS (2000). Multiple comparisons were carried out using the Tukey-Kramer method.

3. Results and Discussion

3.1. Mean Rank of Bacterial Counts by Treatment

Table 2 shows the mean rank of bacterial counts according to species and treatment. The concentration of SP, bacterial species and their interaction had a significant effect ($P<0.05$) on the bacterial counts.

Species	N	Treatment (% Concentration of *SP*)	Mean rank of Bacterial counts	SD	Minimum	Maximum
S. agalactiae	4	Control (Commercial)	0.00	0.000	0.00	0.00
	4	20	36.75	4.349	33.00	41.00
	4	40	32.25	4.924	28.00	37.00
	4	80	29.88	4.090	25.50	35.00
	4	100	25.63	3.065	23.50	30.00
S. aureus	4	Control (Commercial)	0.00	0.000	0.00	0.00
	4	20	33.25	13.598	21.00	46.00
	4	40	43.00	5.228	38.00	48.00
	4	80	34.13	11.346	18.50	43.00
	4	100	15.88	3.966	12.00	20.00
E.coli	4	Control (Commercial)	0.00	0.000	0.00	0.00
	4	20	22.75	14.863	14.00	45.00
	4	40	11.25	3.403	8.00	16.00
	4	80	6.50	2.082	4.00	9.00
	4	100	2.75	1.708	1.00	5.00

Table 2: Mean rank of bacterial counts by treatment
**SP denotes Schkuhria pinnata, SD is the standard deviation*

Increasing the concentration of Schkuhria pinnata reduced the mean rank of bacterial counts. E. coli was most sensitive to the medicinal plant; whilst S. agalactiae was least sensitive. However, the plant extracts were not as effective as commercial control which recorded nil counts.

3.2. Effects of Schkuhria pinnata Concentration on Bacteria Count

For all bacterial species, counts decreased with increasing concentration of Schkuhria pinnata extract although decreases were more pronounced for E. coli than for the other two species. Treatment with 20% concentration of Schkuhria pinnata had significantly higher bacterial counts than the other three treatments ($P<0.05$), but there was no significant difference ($P>0.05$) at 40, 80 and 100% concentration (Table 3). This was expected because for most drugs used in control of diseases, effectiveness increases with concentration.

Treatment (% Concentration of Schkuhria pinnata)	LS mean bacterial count rank	Standard error
Control (Commercial)	0.00^a	0.00
20	30.75^b	2.105
40	28.79^b	2.105
80	23.45^b	2.105
100	14.80^c	2.090

Table 3: LS Mean ranks of bacterial counts by concentration
**Values with different superscripts within column are significantly different ($P<0.05$).*

The relatively higher counts obtained for the plant extracts when compared to the commercial antibacterial agent could be attributed to a number of things including insufficient knowledge of effect of the stage of harvesting of Schkuhria pinnata on potency of the active ingredient and knowledge of the mode of its action. The type and level of biological activity that can be exhibited by any plant material depends on many factors, including the plant part, geographical source, soil conditions, harvest time, moisture content, and post-harvest processing (Chitra et al., 2012). Earlier research showed that the plant parts that were more frequently used to prepare ethno-veterinary medicines were the leaves, barks, roots, seeds and fruit (Toyang et al., 2007; Monteiro et al., 2011; Muhammad et al., 2012); but the use of the whole plant as in this study was rarely reported. According to Maphosa and Masika (2010) plants tend to yield their highest healing potential at peaks of flowering and blossoming, while rhizomes and tuberous roots are most effective before flowering. Moreover, in plant preparation stage, relatively high temperatures that can be generated during tissue grinding can denature chemical constituents, while extraction solvent, and time period can affect level and composition of secondary metabolites extracted from plant tissues (Chitra et al., 2012).Therefore detailed physiological and plant preparation knowledge for Schkuhria pinnata plant is needed, as the plant should be collected at the right time, and in the right way.

3.3. Effect of Type of Solvent on Bacterial Counts

The type of solvent had no effect ($P>0.05$) on bacterial counts. This is primarily implying that the Schkuhria pinnata juice could be extracted using either of the available solvents with the same results. It is therefore advantageous for Schkuhria pinnata juice to be commercially extracted using water as a solvent; as this is freely available to smallholder farmers. Moreover, caution has to be taken when using methanol as a diluent commercially because of its negative effect on animal skin. Methanol may be absorbed through intact skin, and may cause adverse reproductive and foetal effects in animals (Pritchard, 2007; Brito et al, 2012).

3.4. Treatment Effects by Bacteria Strain

Generally the plant extracts appeared to have a greater influence on *E. coli* because of the relatively low counts for the strain (Figure 1).

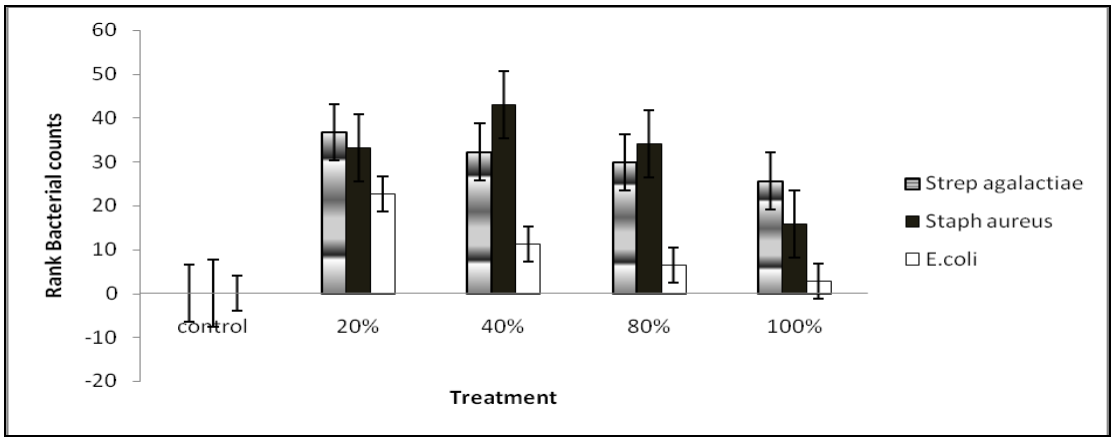

Figure 1: Comparison of treatment by bacterial strain

Comparing by strain, E. coli had significantly lower ($P<0.05$) bacterial counts than S. aureus and S. agalactia implying that it was easily affected by the treatment (Table 4).

Bacterial spp.	LS Mean rank of bacterial count	Standard error
E.coli	10.97^{a}	1.977
S. aureus	31.35^{b}	1.891
S. agalactiae	31.13^{b}	1.794

Table 4: LS mean rank bacterial counts by bacterial strain

The difference in sensitivity between gram-positive bacteria (S. aureaus and S. agalactiae) and gram-negative bacteria (E. coli) could be linked to the structural and compositional differences between the two groups of the bacterial strains (Chitra et al., 2012). Characteristically gram-positive bacteria have thick and tough layer (multilayered) and are highly resistant to physical disruptions, while gram-negative bacteria have thin layer (single layered) and have low resistance to physical disruptions (Dego et al., 2002). Thus, the coat of E. coli bacteria may have facilitated action by the Schkuhria pinnata extract.

3.5. The Effect of Interaction between Treatment and Bacterial Species on Bacterial Counts

The interaction between treatment and bacterial species significantly influenced bacterial counts ($p<0.05$). Figure 2 shows the interaction effects for the two variables.

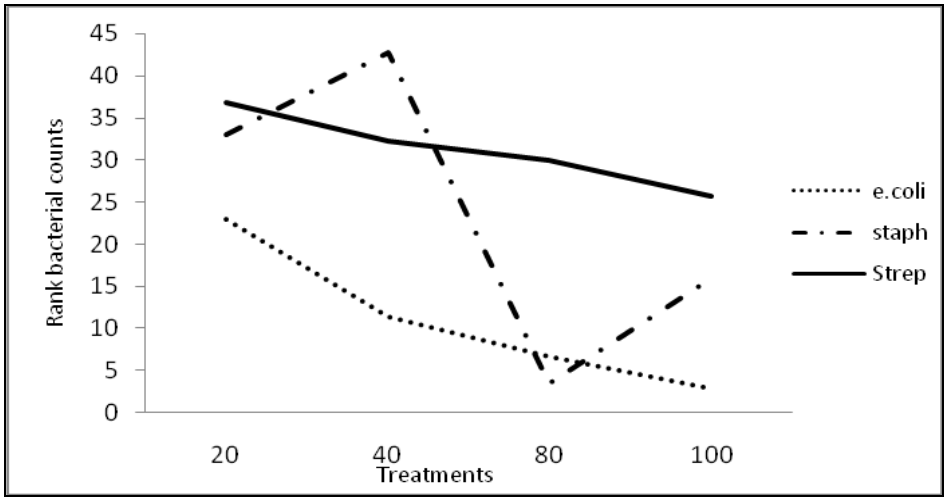

Figure 2: Effect of treatment on three bacterial species

The results indicated a gradual decline in bacterial counts with increasing concentration of the plant extract for E. coli and S. agalactiae. S. aureus rank bacteria counts declines were drastic from 40 to 80% concentrations; while from 20% to 40% and 80% to 100% concentrations tends to increase. Generally, interactions indicated that for the three bacterial strains, counts decreased significantly with increasing concentrations of the Schkuhria pinnata plant extract. This has been observed in other studies where plant extracts of Artemisia dracunculus (Raiesi et al., 2012), Warbugia ugandensis (Njire et al., 2012), Humus lupus, Mahonia aquifolium, Usnea barbata (Chitra et al., 2012), Azadirachta indica (De and Mukherjee, 2009) were used against gram-negative and gram-positive bacterial strains. The increase of S. aureus rank bacterial counts at 40% and 100% concentrations could be either a methodological discrepancy of bacterial test strains used (Njire et al., 2012) or efflux pumps in the bacteria which expel a wide spectrum of structurally unrelated anti-microbial (Veen and Konnings, 2007). This requires verification from further studies.

From this preliminary study, we conclude that plant extracts from Schkuhria pinnata can potentially be effective in the control of mastitis pathogens under laboratory conditions. Further research to identify the bioactive components, plant part to be used, dosage and efficacy in vivo is needed if the mastitis burden can be alleviated.

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