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Article

Phytochemical Screening and Effects of Slerocarya Birrea Caffra (Marula) Stem Bark Extracts on Escherichia Coli Which Causes Diarrhoea in Eswatini

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Abstract: Marula stem bark is used by most Africans in the villages including Swazis in the treatment of diseases associated with bacterial infections such as *Escherichia coli* and *Shigella* which cause diarrhea. The study aimed at identifying the chemical constituents of *Slerocarya birrea caffra* (marula) stem bark and testing the effectiveness of its extract on *E.coli*. Marula stem bark was crushed and macerated in methanol and distilled water, then filtered to obtain a clear dark orange and light orange extracts. Both aqueous and methanol extracts were analysed qualitatively for secondary metabolites. The results showed presence of alkaloids, flavonoids, saponins and tannins in both aqueous and methanol extracts. For the antibacterial activity study on *E.coli,* each extract had the concentrations; 100%, 50%, 25%, 12.5% respectively. *E.coli* was cultured in Macconkey media, then MHA was prepared and poured onto culture plates which were left for 24 hours in an incubator. After 24 hours, no bacterial growth was found in the methanol extract in all the concentrations yet there were 12 CFU in 12.5% concentration of the aqueous extract.

Keywords: Slerocarya birrea; marula; Escherichia coli; shigella; diarrhoea; Eswatini

1. Introduction

Diarrheal disease is the second leading cause of death in children under five years old [1]. Fortunately diarrhea is both treated and preventable. Diarrhea is defined as the passage of three or more loss or liquid stools per day or more frequent passage than normal for an individual, it is usually a symptom of an infection in the intestinal tract, which can be caused by a variety of bacterial, viral and parasitic organisms [2]. Infection is spread through contaminated food or drinking-water, or from person to person as a result of poor hygiene. Diarrhea can last several days, and it is dangerous because it can leave the body of the infected individual without water and salts necessary for survival [3]. In the past, for most people, severe dehydration and fluid loss were the main causes of diarrhea deaths. Now other causes like septic bacterial infections are likely to account for an increasing proportion of all diarrheal –associated deaths. Children who are malnourished or have impaired immunity as well as people living with HIV are most likely to be at risk of life-threatening diarrhea. Diarrhea is a major cause of morbidity and mortality in rural communities in the continent of Africa particularly in children under the age of five. This calls for the development of cost effective alternative strategies such as the use of herbal drugs in the treatment of diarrhea in these communities. Expenses associated with the use of orthodox medicinal plants have generated re+newed interest and reliance on indigenous medicinal plants in the treatment and management of diarrheal infections in rural communities. The properties of many phenolic constituents of medicinal such as their ability to inhibit enteropooling and delay gastrointestinal transit are very useful in the control of diarrhea, but problems such as scarcity of valuable medicinal plants, lack of standardization of methods of preparation, poor storage conditions and incertitude in some traditional health practitioners are issues that affect the efficacy and the practice of traditional medicine in rural African communities [4]. This review appraises the current strategies used in the treatment of diarrhea according to the western orthodox and indigenous African health-care systems and points out major areas that could be targeted by health –promotion efforts as a means to improve the management and alleviate suffering associated with diarrhea. Community education and research with indigenous knowledge holders on ways to maximize the medicinal potentials in indigenous plants could improve diarrhea management in Africa rural communities. It

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is the most common clinical manifestation of gastrointestinal diseases and can be caused by both infectious and non-infectious agents.

Worldwide, an estimated nine million children, most of them younger than the age of five die annually as a result of diarrhea [5]. It is estimated that diarrhea kills more young children around the world than other diseases like malaria, Acquired Immune Deficiency Syndrome (AIDS) and Tuberculosis (TB). Globally, there are nearly 1.7 billion cases of childhood diarrheal diseases every year. In some rural parts of the developing world, the mother's knowledge on the predisposing factors of diarrhea are poor and at times the frequent occurrence of childhood diarrhea is wrongly perceived as a development stage of the child and at times virtually results in mortality. Diarrheal disease account for 1 in 9 child deaths worldwide, making diarrhea the second leading cause of cause of death among children younger than the age of five. Despite the global decline in death rates of children younger than five years old, the risk of a child dying before turning five years of age remains highest in the WHO African region. The problem of child death in Ethiopia is worse , with an Ethiopian child being 30 times more likely to die by his/her fifth birthday than a child in western Europe. In Eswatini a tenant surveillance was done in Mbabane Referral government hospital in 2013 whereby a total of 33 children under the age of five years with acute diarrhea were admitted at the hospitals and 302 cases had stool samples tested for rotavirus antigens.

Despite all the efforts and interventions carried out on diarrheal disease in the world up to the country Eswatini. Global campaigns have been done to fight diarrhea as the second deadliest illness for children. The following strategies have been done to reduce or eliminate the high rate of diarrheal outbreak; Fluid replacement to prevent dehydration, Zinc treatment, Rotavirus and measles vaccination, promotion of early and exclusive breastfeeding and vitamin A supplementation, promotion of hand washing with soap, improved water supply quantity and quality including treatment and safe storage of household water as well as community wide sanitation promotion. Diarrhea remains the second deadliest disease in Eswatini killing children under the age of five. Findings have shown that 91% of infected children are less than 2 years of age with the highest prevalence (44.7%) between 6-11 months old. These findings advocate the need for introduction of new medication to eradicate this disease for example use of herbal plants like the Slerocarya birrea (Marula). On another note, in Eswatini, there is high rate of drug resistance on antibiotics, side effects and cost of treatment. The methanolic extracts from Slerocarya birrea leaves, roots, barks and kernel oil cake were examined for radical scavenging capacities and antioxidants activities. Medicinal remedies prepared from indigenous plants are almost always the only readily accessible and affordable therapies for the control of diarrhea in developing countries. Medicinal plant are a source of hope for African Rural dwellers who most of the time cannot meet the ever increasing cost of modern allopathic medicine.

The study in treatment of diarrhea using herbal anti-diarrheal constituents of Slerocarya birrea will ginger interest in the area of childhood health and disease in Eswatini [6]. Eswatini especially in rural areas might benefit from the result of the study as it can provide with appropriate information on the concept, signs and symptoms, modes of transmission and the preventive measures of diarrhea using cheap and easily available herbal plant. The findings will be useful to the public who are having phobia for going to health facilities. Researchers as well might benefit from this study. This study will add to the existing literature in the field on use of herbal plants to eradicate diarrhea. The findings on different constituents present in Slerocarya birrea useful in treating diarrhea will benefit Pharmacist in the field more especially those specializing on drug formulations.

2. Materials and Methods

In this study, a quantitative, experimental method was used. The bark of Slerocarya birrea was collected from the Shiselweni region in Eswatini where it is mostly habituated. Species identification and authentication was done at Eswatini National Herbarium. Test bacteria was received from ASD Pharmaceuticals and carefully stored in agar plates in Macconkey media for the antibacterial study. All other chemicals used were reagent grade.

2.1 Phytochemical screening of S. birrea stem bark and preparation of extracts

The bark of S. birrea was rinsed, and dried in air. After drying it was powdered using a pestle and mortar and was successively extracted with distilled water and methanol at 20°C for 48 h. After removing the solvents at reduced pressure in a rotatory evaporator, extracts of each plant organ were analysed qualitatively for

secondary metabolites, namely alkaloids, anthranoids, flavonoids, glycosides, polyphenols, saponins, steroids and tannins using standard methods. The crude extracts were stored in a refrigerator at -4°C in sealed glass bottles until further use as shown in Fig. 1.

2.2 Antibacterial study

The Miles and Misra Method was used to study the effect of the marula extract on E.coli test bacteria. Serial dilution of the inoculums/suspension was done in which the dilution of 1x suspension was added to 9x of diluent. The average of three plates was calculated. This was required to have greater assurance of results. All three plates were inoculated with each dilution. A 20 µl drop was absorbed in the plate after 15-20 minutes of continuous spreading by natural means or rotation of plates. All the plates were equally divided into up to eight sectors. In each sector, 1 x 20 µl of the dilution was dropped onto the surface of the agar and thus the drop spread. The plates were kept upright to dry before inversion and incubation at 37°C for 18 to 24 hours.

Each sector was observed for growth, luxurious growth was observed at high concentrations over the area of the drop, or a large number of small colonies which are generally merged. Colonies were counted in the sector where the highest number of full-size discrete colonies were seen. The following equation was used to calculate the number of colony forming units (CFU) per ml from the original aliquot / sample:

CFU per ml = Average number of colonies for a dilution × 50 × dilution factor.

Fig. 1. Crushing of the stem bark in process.

A single isolated colony of bacteria was removed from tryptase soy agar plates and grown separately in 10 mL of tryptase soy broth for 24 hours at 37°C. After incubation, the tubes were centrifuged for 20 minutes at 2,000 rpm. The cell pellets were washed with 10 mL of tryptase soy broth. Then bacterial suspensions in tryptase soy broth were adjusted to the McFarland 0.5 standard.

One µL of bacterial suspension was added to 9 µL of the antiseptic or disinfectant solutions at room temperature for a contact time of 30 seconds, 1.5 minutes, 5 minutes and then 1 µL was removed to 9 µL of the neutralization system and serially diluted. One µL of each dilution was placed onto 3 tryptase soy agar plates by the spread-plate technique and incubated at 35°C for 18 to 24 hours. Surviving colonies were enumerated and expressed as colony-forming units per milliliter.

Concerning the forest service offices we considered if the plant was not stressed due to drought or other disturbances. We took the proposal to the Pharmacy department for approval. Then to the EMCU Ethics committee. Then finally to the national Health Research Review Board in the Ministry of Health in Eswatini. Before we began the experiments in the laboratories we asked for a permission letter from the head of Pharmacy department to use the laboratory as well as from the head of Medical Laboratory Science department.

The reduction rate was calculated as the expression of the disinfectant efficacy, according to the following formula: $\log_{10}$ reduction = $\log_{10}$ pre-disinfection count – $\log_{10}$ disinfection count. By subtracting the logarithm of the former from the logarithm of the latter, the decimal log reduction or microbicidal effect (ME) is obtained. An ME of 1 equals to a killing of 90% of the initial number of bacteria, an ME of 2 means 99% killed. A generally accepted requirement is an ME that equals or is greater than 5: at least 99.999% of the germs are killed. Log_{10} reductions of 5 or more will be taken as an indication of satisfactory microbicidal activity. All

plates were disinfected, sterilized or pressure-sterilize (autoclave) in a heat-stable biohazard bag for safe disposal.

3. Results and Discussion

The results are presented in Table 1. Since we were supposed to also test the marula stem bark extract on Shigella which also causes diarhhoea in Africa including Eswatini, in short the table shows the effectiveness of marula stem bark extract only on E. coli, but the extract was both aqueous and methanol extracted from the Pharmacy Laboratory. There is no data to present the results from the Pharmacy laboratory where the extraction was carried out, because not much was done like photochemical analysis of the extract but it was only extraction which included crushing of the bark, maceration, and filtration of both preparations (methanol extract and aqueous extract). Figs. 2 - 6 were taken from the Pharmacy laboratory.

Table 1. Antimicrobial activity of marula stem bark on E. coli (CFU/ml)

Marula stem bark method extract				Marula stem bark aqueous extract			
100%	50%	25%	12.5%	100%	50%	25%	12.5%
0	0	0	0	0	0	0	12

Fig. 2. E. coli grown in macconkey media.

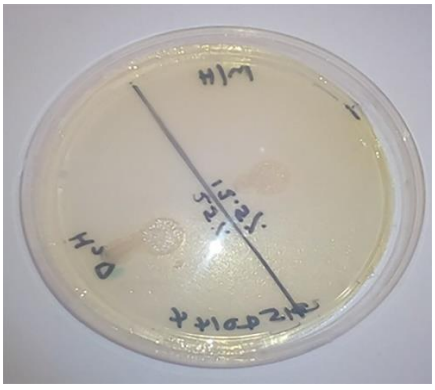

Fig. 3. Petri dish showing the effectiveness of the extract.

Fig. 4. Crushed marula stem bark.

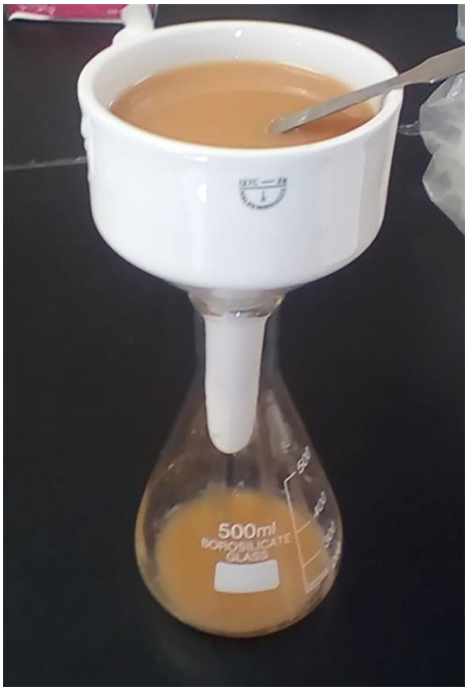

Fig. 5. Filtration of the solution after maceration.

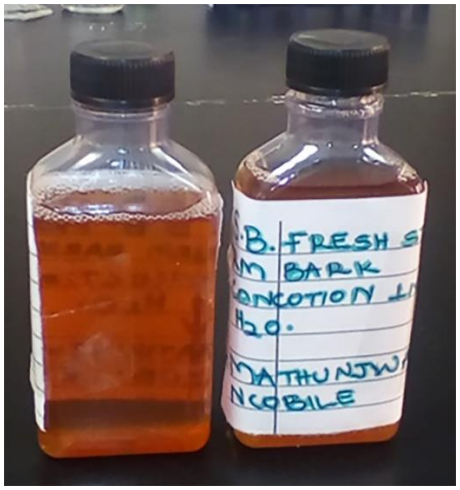

Fig. 6. The final aqueous and methanol extract.

Marula stem bark methanol extract indicated some effectiveness from the concentration of 100% to 12.5% compared to the aqueous extract that did not show effectiveness in 12.5% concentration. Several previous

studies agree with the results above. A study was carried out and reported ten plant species most widely used to treat diarrhea in South Africa and Zimbabwe and it was discovered that of the lot. Marula tree was the most popular medicinal plant used as an antidiarrheal remedy and on top of it all the bark was found to be the second frequently used part of the marula tree by 26.3%. In addition, the bar chart which presented the results showed that Slerocarya birrea is the most widely used and effective plant species for the treatment of diarrhea. Marula bark is widely used for bacteria-related diseases by indigenous cultures in Africa, and Eswatini is also in Africa. In the study bark and leaves were extracted with acetone and MIC values were determined using a micro plate serial dilution technique which we also used to test for the effectiveness, S. aureus, P. aeruginosa, E. coli, and Enterococcus faecalis were used as test organisms. All extracts were active with MIC values from 0.15 to 3 mg/ml. Based on minimum inhibitory concentration values, inner bark extracts tended to be the most potent followed by outer bark. Marula stem bark was one of the plants he was investigating to test if they treat diarrhea caused by bacteria more especially E. coli. He found that the bark of S. birrea is the part most frequently used to treat ailments that are mostly bacteria-related, the ailments include stomach-aches, diarrhea, wounds and coughs, therefore we was so concerned about the diarrhea which the study also is concerned with since infectious diarrhea is the second largest single cause of mortality in children the age of five in Eswatini and bacteria are responsible for most diarrheal episodes especially in developing countries like Eswatini, and progressive increase in antimicrobial resistance has given rise to the need to investigate other sources of therapy such as the medicinal plants remedy. It was investigated as a drunk decoction. Phytochemical screening and antibacterial activity of stem bark, leaf, and root extract of Slerocarya birrea were reported. Preparation of the plant extract was carried out in a laboratory. Phytochemical tests were done as well as antibacterial activity assays, also in this study methanol was also used for extraction of plant, since the most studies have reported that organic solvents were better chemical reagents for consistent extraction of antimicrobial substances from medicinal plants. The presence of tannins, flavonoids and saponins was proved in the bark of marula tree extract of methanol, yet in this study we didn't do the phytochemical screening because it was already done in Eswatini. It was found that there are no alkaloids present in the bark of S.birrea, which means that these are the compounds that are effective against E. coli. Manzo et al also found out the results of antibacterial sensitivity test of S. birrea against E. coli and upon looking at their results under the bark zone of inhibition there were bigger figures compared to leaves and roots of the same plant, the zone of inhibition was 13 mm on ethanol extract and 17 mm on methanol extract, which clearly proves that the results are correct since we used the same solvent. On the same study, the minimum inhibitory concentration of methanol extract of S. birrea against E. coli was present from 50 mg/ml up to 12.5%. And comparing it to the results which said that there was no growth of E. coli in all concentrations of methanol extract of s. birrea but there was growth only at 12.5% concentration of the aqueous extract. And on another note we decided to also use water because in the villages traditional healers use water for their medication. Looking at all the previous studies done by different scientists, the most effective part of the marula tree in treating ailments that include diarrhea caused by E. coli is the bark of marula tree. Moreover the methanol extract is the most effective although in the villages they just use aqueous extractrion.These all prove that indeed Marula stem bark is indeed effective against diarrhea caused by bacteria such as E. coli.

At first when we started these research project we wanted to test the marula stem bark extract in E. coli and Shigella which both causes diarrhea. Unfortunately after researching more on the topic we discovered that it is dangerous to collect the data using shigella. Beside that we discovered that its access is very difficult and having shigella in an environment, it can bring chaos since everyone can be infected easily, just its presence.

Gastrointestinal disorders, diarrhea in particular have remained a major concern in Southern African countries including the small country Eswatini. The study aimed at proving and testing if Slerocarya birrea (marula stem bark) extracted with methanol and another one extracted with water is effective against E. coli which is one of the bacteria that causes diarrhea in Eswatini. Marula tree has so many parts each with its medicinal importance, the fruit itself has vitamin C therefore it offers a protection against Scurvy. The fruit also contains potassium, calcium and magnesium. The skin of the fruit can be boiled to make a drink or burnt to be used as a substitute for coffee; the green leaves are eaten to relieve heartburn. The nuts are rich in protein which is good for a healthy human being. It has been also found that the piece of bark can also be crushed into a pulp, mixed with cold water and swallowed in the treatment of dysentery and diarrhea. The bark also is used as a malaria prophylactic. Among the many strains of E. coli, only a few trigger diarrhea, one group of E. coli which includes 0157:H7 produces a powerful toxin that damages the lining of the small intestine, which can cause bloody diarrhea. You develop an E. coli infection when you ingest this strain of bacteria. Marula stem bark is a

good remedy for treating minor ailments associated with bacteria more especially E. coli. The bark has secondary metabolites that can kill the bacteria after being taken orally or anally (enemas) by an infected individually. Clean and sterilized materials have to be used during the extraction to avoid contamination.

After obtaining the results after the study, we can recommend that traditional healers continue treating their patients with diarrhea using marula stem bark decoction in aqueous extraction but the results push us to recommend that mostly to be sure that their medicine is really effective they can use methanol as a solvent for extraction.

In conclusion, based on literature and results of this study, S. birrea tree has its different parts each with medicinal importance; the fruit has vitamin C therefore it offers a protection against scurvy. The fruit also contains potassium, calcium and magnesium. The skin of the fruit can be boiled to make a drink or burnt to be used as a substitute for coffee; the green leaves are eaten to relieve heartburn; the nuts are rich in protein which is good for a healthy human being. It has been also found that the piece of bark can also be crushed into a pulp, mixed with cold water and swallowed in the treatment of dysentery and diarrhea. The bark also is used as a malaria prophylactic.

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