In vitro Antibacterial Effect of *Combretum molle* and Fr1 against *Staphylococcus aureus* and *E. coli* Isolated from Bovine Mastitis

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**ABSTRACT**

*In vitro* antibacterial sensitivity test of selected medicinal plants was conducted at AAU, from December 2014 to May 2015. The study carried out with the objective of determining and comparing of the *in vitro* antibacterial effect of ethanol and methanol extracts of FR1 leaf and C.molle seed which is sequentially extracted with petroleum ether, acetone, ethanol and methanol at various concentrations against *Staphylococcus aureus* and *Escherichia coli* isolated from bovine mastitis cases using agar disc diffusion technique. The plants for this study were selected based on previous research works and collected from their natural habitats. The leaf of FR1 were processed and extracted by 95% ethanol and methanol. In case of C.molle seeds, Sequential extraction method was performed in order to get different alcohol soluble fraction.

In this study, both plants inhibit the growth of *S. aureus* and *E. coli* at all concentration (4mg/disc to 0.25mg/disc) except FR1 leaf at 12.5mg/ml (0.25mg/disc) had no antibacterial effect on *E. coli*. Both types of plants were a dose dependent inhibition zone on the tested bacteria showing greatest activity at highest concentration of crude extracts. The absolute methanol extract of FR1 showed a good inhibitory effect on test organisms than its ethanol extract.
A wider zone of inhibition was observed on ethanol soluble fraction of C. molle seed against E.coli. Antibacterial activities of both 200mg/ml methanol extract of FR1 and 200mg/ml of acetone soluble fraction of C. molle against S. aureus was higher than the activity of the standard antibiotic (Ampicilin) with mean zone of inhibition 16.33 and 18.33 respectively. Also 200mg/ml ethanol soluble fraction of C. molle shows significantly comparable antibacterial activity against E.coli with that of the standard antibiotic (Ampicilin) with mean zone of inhibition 19.33. From this study, we can conclude that type of solvents used matter for the efficacy of the plant/herb. The efficacies of 200mg/ml crude extract of both plants suggest that there potential in the discovery of novel antimicrobial agents from medicinal plants and further study should be made in order to identify the active phytochemical constituents and on toxicity of active plant principles to determine their safety use.

Keywords: In-Vitro Antibacterial Effects, Crude Extracts, Medicinal Plants, Staphylococcus aureus, E. coli and Zone of inhibition.

INTRODUCTION

Bovine mastitis has remained the most economically damaging disease that severely reduces milk production and often difficult to treat due to antimicrobial resistance (Getahun et al., 2008). In addition to heavy losses in milk quality and quantity, it also causes irreversible damage to the udder tissue and less occasional fatalities (Radostits et al, 2000). Many pathogens can cause mastitis, but the majority of IMI is caused by a few bacterial species. The most important major pathogens involved with bovine mastitis worldwide are Staphylococcus aureus, Streptococcus uberis, Streptococcus agalactiae, Streptococcus dysgalactiae, Escherichia coli and Klebsiella species (Olde et al., 2008). In Ethiopia, the available information indicated that bovine mastitis is one of the most frequently encountered diseases of dairy cows (Abunna et al., 2013).

Successful treatment depends on a specific and accurate diagnosis, followed by treatment with appropriate antibacterial product (Thomson et al., 2008). The conventional drugs used for the treatment of mastitis are limited in types in developing countries in general and in Ethiopia in particular. Due to this and other factors the causative agents were also reported to have developed variable degrees of resistance to the commonly used antimicrobial agents. It is therefore important to design and follows strategies that could help in the utilization and development of locally available low cost indigenous resources (Tolosa et al., 2010).

The extensive use of antibacterial agents over the last 50 years has led to the emergence of bacterial resistance and to the dissemination of resistance genes among pathogenic microorganisms (Chambers, H. F. 2001). Presently, one of the greatest hazards regarding health preservation in human and veterinary medicine is the declining chance of success full anti microbial therapy due to resistance (Salisbury et al., 2002). The emergence of drug resistance is an evolutionary process that is based on selection for organisms that have an enhanced ability to survive and reproduce in the presence of the drug (Suleiman et al., 2010).

Nature has a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, based on their use in traditional medicine. It is reported that over 50 percent of all modern clinical drugs are natural product origin and natural product play an important role in the drug development programs in the pharmaceutical industry (Bharathi et al., 2010). Traditional healers have long used plants to prevent or cure infectious conditions. Western medicine is trying to duplicate their successes (Lewis K. and Frederick M., 2006).
Traditional medicine has been defined as health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises to treat, diagnose and prevent illness or maintain well being (WHO, 2001). Since ancient times plants have been indispensable sources of both preventive and curative traditional medicine preparations for human beings as well as livestock (Lulekal, 2008). In Ethiopia, Modern livestock health care is still at its lowest stage due to lack of adequate clinics, veterinarians and supply of drugs. Besides, most modern drugs are expensive and, as a result, not affordable by the majority of Ethiopian farmers and pastoralists. As a result, people rely on their traditional knowledge, practices and locally available materials, mainly plants, in the management of diseases of their domestic animals (Giday M. and Ameni G., 2003).

Out of the total flowering plants reported from the world, more than 50,000 are used for medicinal purpose (Schippmann et al., 2002). In Ethiopia, plant remedies are still the most important and sometimes the only sources of therapeutics for nearly 80% of human and more than 90% in livestock population. Estimated floras of 6500 to 7000 species of higher plants are of medically important and out of these medicinal plants 12% are endemic to Ethiopia (Giday et al., 2009). Many works that document the wealth of indigenous knowledge on the ethno biology and ethno medicine have been emerging. However, very few information exists on the veterinary herbal medicines (Sori T.et al., 2004). Since most traditional knowledge in Africa in general and in Ethiopia in particular is transmitted orally, from generation to generation, knowledge of wild plants is in danger of being lost as habitats, value systems and natural environments change (Yineger H. and Yewhalwa D., 2007; Mesfin et al., 2009). Thus, valuable indigenous knowledge associated with medicinal plants warrants proper documentation (Awast T. et al., 2012).

In Ethiopia, traditional healers use a number of plants/herbs for the treatment of bovine mastitis. The efficacies of some of these plants/herbs have been tested against a range of causative agents of mastitis. Taddese (2007) has conducted in vitro tests of C. molle on S. aureus isolate. Regassa, F.and Mengistu, A. (2012) conducted in vitro antimicrobial activity of Combretum molle (combretaceae) against Staphylococcus aureus and Streptococcus agalactiae. Sahlu (2013) has also conducted study on antibacterial activities and preliminary phytochemical investigation of four selected medicinal plants namely leaves, steam, bark and seeds of C. molle, steam bark of Bereza and leaves of Xanthium strumarium and Laggoria arota against Staphylococcus aureus, Streptococcus agalactiae and Escherichia coli. Kinde (2014) has conducted an in vitro test of X. strumarium, C. molle and FR1 on S. aureus and S. agalactiae isolates and observed encouraging result.

Therefore the main objectives of the present study are;

- To make comparison of the yield and antimicrobial activity of ethanol and methanol extracts of FR1 leaf and the seeds of Combretum molle extracted successively with different solvents and the in-vitro antimicrobial effects of two phytopreparation; namely C. molle and FR1 on Staphylococcus aureus and Escherichia coli isolated from bovine clinical mastitis case.

**MATERIALS AND METHODS**

**Study Area**

A study of an in-vitro antimicrobial effect of phytopreparation was carried out at Bishoftu between December 2014 and May 2015. Bishoftu town is located at 47 km South-east of Addis Ababa. The area has an altitude of 1,860 meters above sea level with an average
annual rain fall of 866 mm. It has a bimodal rainy seasons; a main rain season extends from the month of June to September and a short rainy season from March to May. The annual average minimum and maximum temperature is 11 °C and 26 °C, respectively. Day length is fairly constant throughout the year (12-13 hrs) with about 6 hours of sunshine during the rainy season and 8 to10 hours for the rest of the year. Humidity is about 50.9 % (Mungube, 2001).

Figure 1.

Study Design
An experimental trial study on in-vitro antimicrobial efficacies on selected plants was conducted between December 2014 and May 2015 in AAU, CVMA, Bishoftu. During the study period the efficacy of the dry seeds of Combertum molle directly collected from the trees from north Gondar Weyroch wereda Qoqma PA and FR1 leaf collected form Bishoftu area against the test organisms was compared using antibacterial activity on disc diffusion assay. Finally, the antibacterial activities of different solvent extracts with highest efficacy were compared with those of the standard antibiotic (Ampicilin).

Herbal material used for the study
Combertum molle (“Agalo, Abalo” in Amharic, “Bika, Dadamata” in Oromiffa) :this is a member of the family Combertaceae which is small deciduous tree growing up to 15 meters high with an often-crooked trunk, commonly branching to the base. The bark is dark brown to black and deeply grooved in squares. The leaves are oppositely arranged, elliptic to lanceolate, large that covered with soft hairs, rounded at the base. Flowers sweetly scanted, many crowded into greenish. The flowers generally appear before the leaves and the fruits yellowish, four-sided with wings (Mengistu, 2004). The seeds were used to assess the antimicrobial effect on bacteria isolated from mastitis case.

Unidentified plant (coded FR1): It is a type of plant found dispersed in many areas in Bishoftu with a height of up to 1.5 meters and branched at the base and the leaves are wide. This plant selected based on its use locally for the treatment wound. It was collected in Bishoftu city and was used for the in-vitro antibacterial test.

Bacterial organisms used for the study
Two species of bacteria, S. aureus and E.coli isolated from the clinical mastitis case was used for testing bacteria. These organisms were selected because they are among many pathogens often implicated in bovine mastitis case. These bacteria were streaked onto
nutrient agar to obtain pure isolated colonies, following a standard aseptic technique and the four-way streak plate inoculation (Cappuccino and Sherman 2008).

Study Methodology

**Plant collection and pre extraction preparation**

The seed *C. molle* plant samples were obtained from Gonder and chosen based on the results reported by previous research works (Taddese, 2007). The leaf of FR1 were collected from college of veterinary medicine and agriculture, Bishoftu. After collection, the plants were washed with tap water to remove unnecessary particles. Then air dried under shade and ground in to powder using pestles and wooden mortars. The powdered materials were then sieved and stored in labelled airtight bottles at room temperature until used.

**Extraction Solvents used for the study**

Solvents differ in their extraction capabilities depending on their own chemical properties and the solute’s chemical structures. Other factors affecting solvent selection are boiling point, density surface tension, viscosity, corrosiveness, flammability, toxicity, and stability, compatibility with product, availability, and cost (Cowan, 1999). Many types of solvents are available for extracting plant materials including ethanol, methanol, acetone, and petroleum ether. Most widely used solvents are water and ethanol. For this study petroleum ether, acetone, methanol, and ethanol were successively used for extracting *C.molle* seed and ethanol and methanol were used for extracting FR1 leaf (Thabile, 2008).

**Preparation of crude extracts for in-vitro experiment**

**Ethanol and methanol extraction of FR1**

About 50g of the powdered herb leaf of FR1 was macerated in 250ml of 95% Ethanol and methanol in a separate flask for at least 72 h with frequent shakings. The samples were then suction filtered through Whatman No.1 filter paper. The filtrates were evaporated in a rotary evaporator to remove the solvents under reduced pressure at 40°C. The plant extracts were then taken out and put into evaporating sterile Petri dishes and kept in a dry oven at 40°C to remove the remaining solvents for 24 hours. The procedure was repeated in the same way to have sufficient amount of extracts. Finally, concentrated extract was weighed, transferred and labeled with the respective plant names and stored at +4°C in the dark until tested for antimicrobial activity. The percentage yield of each plant extracts are shown in (Table.1 and 2).

**Sequential extraction of seeds of C.molle**

About 47grams powdered seed of *C.molle* was extracted sequentially with petroleum ether, acetone, ethanol and methanol by macerating in the respective solvents (250ml each) for three days. To clarify, the marc (i.e. plant material left after extraction with solvent, and then air-dried) left after extraction with petroleum ether was extracted with acetone. The marc left thereafter was extracted with ethanol, and finally, the marc left after extracted with methanol. This led to the production of four types of extracts, viz. petroleum ether-soluble fraction, acetone-soluble fraction, ethanol-soluble fraction and methanol-soluble fraction from seed of *C.molle*. The rest procedures were carried out in the same way used for ethanol and methanol extracts. A total of four extracts for the plant were thus obtained which were screened for antimicrobial activities.

**Preparation of antimicrobial discs from herb extracts**

The empty antibiotic discs approximately 6mm in diameter were prepared by using ordinary office hole puncher. Before the discs were impregnated they were put into screw capped bottle and autoclaved a 121 °C for 15 minutes. A stock solution of 200mg/ml in 10% Tween 80 was made for each extract.
A serial dilutions with different concentrations of each solvent extracts of FR1 leaf and seed of *C. molle* (200, 100, 50, 25 and 12.5 mg/ml) were prepared using 10% Tween80 as described by (Olila *et al.*, 2001). In the first test tube 2 ml of 200mg/ml of working solution was added and each of the remaining five tubes was filled with 1ml of 10% Tween 80. A milliliter of 200mg/ml solution from the first tube was transferred to a second test tube to prepare 100mg/ml. The procedure continues by transferring 1ml of solution from the 100mg/ml preparation to a third test tube to get a 50mg/ml concentration, and the procedure continued in a similar manner until a12.5 mg /ml concentration is reached. Discs of 6mm diameter were impregnated by adding 20μl from each reconstituted solution 200(4mg/disc), 100(2mg/disc), 50(1mg/disc), 25(0.5mg/disc) and 12.5 (0.25mg/disc) and then the discs were allowed to dry at room temperature in bio safety cabinet level II. After drying they were used for screening the antibacterial activity of the respective plant types.

**Preparation of the test bacteria**

The plants extracts were tested against bovine mastitis bacterial isolates obtained from the Department of Microbiology, college of veterinary medicine and agriculture. These included one Gram positive bacteria: *Staphylococcus aureus*; and one Gram negative bacteria: *Escherichia coli*. The bacterial isolates were maintained on nutrient agar and sub cultured every three days. An inoculum (three well isolated colonies) of each bacterial isolates was suspended in 5 ml of saline solution (0.85%) and adjusted to give a concentration of bacterial cells equivalent to a McFarland No. 0.5 standard (1-2 108) prior to the antibacterial testing.

**The in-vitro antimicrobial sensitivity test**

The antimicrobial test of ethanol extract was conducted using agar disc diffusion method. Muller-Hinton agar (38gm) (Biotech UK) medium was used for antimicrobial sensitivity test, and was mixed with 1 litter of distilled water boiled to dissolve completely and autoclaved at 121 °c for 15minutes. Therefore, the agar was prepared by pouring 25ml in a 90mm sterile agar plates and left to set. The agar plates were incubated for 24 hrs at 37 o c to confirm their sterility. When no growth occurred after 24 hrs, the plates were considered sterile and used for antimicrobial sensitivity tests. The three well isolated colonies of the same morphology were scooped using a wire loop from the nutrient agar and mixed using sterile normal saline, and agitated with a vortex mixer. The turbidity of the bacterial suspension was adjusted by comparing with 0.5 McFarland turbidity standards (1x 10^8 cfu/ml). McFarland turbidity standard was prepared by mixing 0.5ml of 1.175% aqueous solution of barium chloride (0.048M BaCl₂H₂O) with 9.95ml of 1% sulfuric acid (0.036M H₂SO₄). The standard and the test suspension were placed in a 10ml sized tests tubes and compared against a white back ground with contrasting black lines until the turbidity of the test suspension equates to that of the turbidity standard. Adjustments of the turbidity were made by adding saline or colonies depending on the degree of turbidity.

A sterile swab was dipped in to the standardized suspension of the bacteria and excess fluid was expressed by pressing and rotating the swab firmly against the inside of the tube above the fluid levels. The swab was streaked in the three directions over the entire surface of the agar with objective of obtaining uniform inoculations and a final sweep with the swab was made against the agar around the rim of the Petri dish. The inoculated plates were allowed to stand for not more than 15 minutes and the discs were placed on the agar surface using a sterile forceps. Each disc was gently pressed with the point of the sterile forceps to ensure complete contact with the agar surface. The appropriate crude extract impregnated discs and conventional antibiotic discs were applied at spaces of 24mm apart from center to center and 15mm away from the edge of the plates. The plates turned upside down, labeled
and incubated at 37°C for 24 hrs. Ampicillin antibiotic discs (30µg/disc) were used as positive control and the disc with 10% Tween 80 (20µl) was used as negative control. Diameter of ZI was measured using a ruler in mm and results were recorded by comparing with standard values for each conventional antibiotic disc (Ampicillin) (Quinn et al., 1999).

**Data management and Analysis**
Data was checked for its completeness every day. It was edited, cleaned and analyzed: the collected data was entered in to the computer using statistical package for social science (SPSS) version 16. The data was summarized and described using tables and graphs. Descriptive statistics was used to put result of the study in the form of finding Mean and standard deviation.

**ANNEXES**


1.**Gram’s stain**
   **Procedure:**
   1. Make a thin smear
   2. Allow the film to by passing through the bunsen flame several times
   3. Flood the slide with crystal violet for 30-60 seconds
   4. Pour off the stain and wash the remaining stain with iodine solution
   5. Washoff the iodine and shake the excess water from slide
   6. Decolorize with acetone alcohol
   7. Counter stain with safararanin for 30-60 seconds and wash with water
   **Interpretation:** those gram positive bacterias takes the primary stain crystal violet and appear violet or deep purpule, while those gram negative bacterias appear red or pink by taking the color of safaranin.

**Annex 2: Media used for isolation and identification of bacteria (Quinn et al., 1999)**

1. **Mannitol salt agar (Difeo, Detroit, USA)**
   **Composition (gm/l):** Proteose peptone No 3 10.0, Bacto-beff extract 1.0; D.mannitol 10.0; Sodium chloriden 75.0; Bacto agar 10.0; phenol red 0.025.
   **Preparation:** 111 gm suspended in 1 liter distilled water and heat till boiling to dissolve completely. Sterilize in autoclave at 121°C for 15 minutes and cooled to 45-50°C and poured in to petridishes.

2. **Nutrient agar (Oxide, Hampshire England)**
   **Composition (gm/l):** Lamleoco powder 1.0; Yeast extract 2.0; Peptone 5.0; Sodium chloride 5.0; Agar 15.0.
   **Preparation:** suspend 28gm in 5 liter of distilled water bring to boil to dissolve completely and sterilize by autoclaving at 121°C for 15 minutes, pour to petridish after cooling to 45-50°C.

3. **Mueller Hinton media**
   **Composition (gm/l):** Beef Extract 2.0; Acid Hydrolysate of Casein 17.5; Starch 1.5; Agar 17.0.
   **Preparation:** Suspend 38 grams in 1 liter distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes pour to petridish after cooling 45-50°C.
4. Preparation of normal saline (0.9 % NaCl)

✓ 0.9 grams of NaCl was suspended in to 100 ml of distilled water
✓ Sterilized the solution using autoclave at 15lbs pressure (121 °C) for 15 minutes.

5. 0.5 % McFarland turbidity standard preparation

- Solution A:
  - 1.175 grams of BaCl₂.2H₂O was measured
  - 50 ml of distilled water was added and mixed well
  - Made up to 100 ml with distilled water

- Solution B:
  - 99 ml of distilled water was put in to flask
  - 1 ml of concentrated H₂SO₄ was added in to the flask contained distilled water

  0.5 % McFarland turbidity standard= 99.5 ml solution B(1 % H₂SO₄) + 0.5 ml solution A (1.175 % BaCl₂)

4. Eosin Methylene Blue (EMB) (Oxoid, ® Hampshire, England)

Ingredients
Peptone 10.00
Lactose 5.00
Dipotassiummonohydrogen phosphate 2.00
Methylene blue 0.06
Eosin Y 0.40
Agar 13.50
PH 7.1 ± 0.2 @ 25°C

Preparations
Suspend 37.46 grams in 1000 ml distilled water and dissolve and mix thoroughly and gently. Heat to boiling and dissolve the medium completely. Then put into the flask. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. Avoid overheating. Cool to 50°C and shake the medium in order to

RESULTS
Extract yield
Methanol extract of FR1 leaf has higher yield (8.8633 gm) than its ethanol extract (8.2903). Also from serially extracted C. molle seed, acetone gives the highest yield (13.0957 gm), followed by petroleum ether (7.9242 gm), methanol (0.747 gm), while ethanol alcohol was the least with (0.5419 gm)

Table 1. Extract yield of FR1 leaf powder (50gm) extracted with ethanol and methanol.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>1st extraction</th>
<th>2nd extraction</th>
<th>Total mass</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>6.088</td>
<td>2.2023</td>
<td>8.2903</td>
<td>2.551</td>
</tr>
<tr>
<td>Methanol</td>
<td>7.1251</td>
<td>1.7382</td>
<td>8.8633</td>
<td>3.121</td>
</tr>
</tbody>
</table>

The Effects of Crude Extracts on Selected Bacteria
Each plant extracts of the two plant species were tested at different concentration levels (200- 12.5mg/ml) on two species of bacteria. Petroleum ether extract of C. molle has no
antimicrobial effect on test organisms. But other alcoholic soluble fractions (acetone, ethanol and methanol) of *C. molle* showed strong anti microbial activity against *S. aureus* and *E. coli*.

Table 2. Extract yield of *C. molle* seed powder (47gm) serially extracted with four solvents.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Mass residue extracted (gm)</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; extraction</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; extraction</th>
<th>Total mass</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>7.5231</td>
<td>0.6719</td>
<td>7.9242</td>
<td>3.9621%</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>7.2505</td>
<td>5.8452</td>
<td>13.0957</td>
<td>6.5478%</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.3374</td>
<td>0.2045</td>
<td>0.5419</td>
<td>0.2709%</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>0.5906</td>
<td>0.1564</td>
<td>0.747</td>
<td>0.3735%</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Zone of inhibition (mm) of ethanol extract of leaf of FR1 against *S. aureus* and *E. coli*.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Amount per disc</th>
<th><em>S. aureus</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>0.5mg/disc</td>
<td>9±1.0</td>
<td>8.33±0.58</td>
</tr>
<tr>
<td></td>
<td>0.25mg/disc</td>
<td>6.33±1.15</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>30 µg/disc</td>
<td>14±1.155</td>
<td>19±1</td>
</tr>
</tbody>
</table>

Table 4. Mean Zone of inhibition (mm) of Methanol extract of leaf of FR1 against *S. aureus* and *E. coli*.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Amount per disc</th>
<th><em>S. aureus</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>4mg/disc</td>
<td>16.33±0.58</td>
<td>15.67±0.58</td>
</tr>
<tr>
<td></td>
<td>2mg/disc</td>
<td>14.33±0.58</td>
<td>13.33±0.58</td>
</tr>
<tr>
<td></td>
<td>1mg/disc</td>
<td>13±1.0</td>
<td>11.33±1.15</td>
</tr>
<tr>
<td></td>
<td>0.5mg/disc</td>
<td>9.33±1.15</td>
<td>9±1.0</td>
</tr>
<tr>
<td></td>
<td>0.25mg/disc</td>
<td>7±1.0</td>
<td>6.33±0.58</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>30 µg/disc</td>
<td>14±1.155</td>
<td>19±1</td>
</tr>
</tbody>
</table>

Table 5. Mean Zone of inhibition (mm) of acetone soluble fraction of *C. molle* seed against *S. aureus* and *E. coli*.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Amount per disc</th>
<th><em>S. aureus</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>4mg/disc</td>
<td>18.33±0.578</td>
<td>16.0±0.0</td>
</tr>
<tr>
<td></td>
<td>2mg/disc</td>
<td>14.66±0.578</td>
<td>13.66±0.58</td>
</tr>
<tr>
<td></td>
<td>1mg/disc</td>
<td>13.66±1.155</td>
<td>12.33±2.52</td>
</tr>
<tr>
<td></td>
<td>0.5mg/disc</td>
<td>12±1.15</td>
<td>11.33±1</td>
</tr>
<tr>
<td></td>
<td>0.25mg/disc</td>
<td>10.33±1.0</td>
<td>10±1.15</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>30 µg/disc</td>
<td>14±1.155</td>
<td>19±1</td>
</tr>
</tbody>
</table>
Methanol and ethanol extracts of FR1 also shows antimicrobial activity against *S. aureus* and *E. coli*. The alcoholic extracts of the FR1 and *C.molle* inhibited the growth of *S. aureus* and *E. coli* in all concentration (Tables 1, 2) and (tables 3, 4 and 5) respectively except 12.5mg/ml (0.25mg/disc) concentration, ethanol extract of FR1 leaf had no antibacteri al effect on *E. coli*.

**Table 6. Mean Zone of inhibition (mm) of ethanol soluble fraction of *C. molle* seed against *S. aureus* and *E. coli.*

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Amount per disc</th>
<th><em>S.aureus</em> MEAN ±SD</th>
<th><em>E.coli</em> MEAN ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>4mg/disc</td>
<td>16.67±0.58</td>
<td>19.33±1.15</td>
</tr>
<tr>
<td></td>
<td>2mg/disc</td>
<td>15.67±1.15</td>
<td>13.33±1.15</td>
</tr>
<tr>
<td></td>
<td>1mg/disc</td>
<td>13± 1.0</td>
<td>11± 1.0</td>
</tr>
<tr>
<td></td>
<td>0.5mg/disc</td>
<td>12 ±1.15</td>
<td>10.66± 1</td>
</tr>
<tr>
<td></td>
<td>0.25mg/disc</td>
<td>10.33±1.0</td>
<td>10 ±1.15</td>
</tr>
<tr>
<td>Ampicilin</td>
<td>30 µg/disc</td>
<td>14±1.155</td>
<td>19±1</td>
</tr>
</tbody>
</table>

**Table 7. Mean Zone of inhibition (mm) of methanol soluble fraction of *C. molle* seed against *S. aureus* and *E. coli.*

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Amount per disc</th>
<th><em>S.aureus</em> MEAN ±SD</th>
<th><em>E.coli</em> MEAN ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>4mg/disc</td>
<td>15±2.0</td>
<td>14± 1.0</td>
</tr>
<tr>
<td></td>
<td>2mg/disc</td>
<td>13± 1</td>
<td>13.33±0.58</td>
</tr>
<tr>
<td></td>
<td>1mg/disc</td>
<td>12 ± 0.0</td>
<td>10± 1.53</td>
</tr>
<tr>
<td></td>
<td>0.5mg/disc</td>
<td>11.33± 1.15</td>
<td>11 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>0.25mg/disc</td>
<td>10.66 ± 1.0</td>
<td>10 ±0.0</td>
</tr>
<tr>
<td>Ampicilin</td>
<td>30 µg/disc</td>
<td>14±1.155</td>
<td>19±1</td>
</tr>
</tbody>
</table>

**Table 8. MZI in mm for the effect of ethanol and methanol extracts of FR1 leaf and acetone, ethanol and methanol soluble fraction of *C. molle* seed herbs compared to positive control (Ampicilin) and negative control (10%Tween 80) on *S. aureus* and *E. coli.*

<table>
<thead>
<tr>
<th>Type of diagnostic discs</th>
<th>Diameter of MZI (mm) on <em>S. aureus</em></th>
<th>Diameter of MZI (mm) on <em>E.coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol extract of FR1 leaf</td>
<td>15.67</td>
<td>14.33</td>
</tr>
<tr>
<td>Methanol extract of FR1 leaf</td>
<td>16.33</td>
<td>15.67</td>
</tr>
<tr>
<td>Acetone extract of <em>C. molle</em> seed</td>
<td>18.33</td>
<td>16</td>
</tr>
<tr>
<td>Ethanol extract of <em>C. molle</em> seed</td>
<td>16.67</td>
<td>19</td>
</tr>
<tr>
<td>Methanol extract of <em>C. molle</em> seed</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Ampicilin</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>Tween 80</td>
<td>NI</td>
<td>NI</td>
</tr>
</tbody>
</table>
Effect of 200mg/ml Herbal Extracts in Comparison with Conventional Antibiotic Discs

In general the size of the diameter of inhibition zone exhibited by 200mg/ml concentration of the plant extracts was found comparable to those of noble antibiotic discs (Ampicilin). 10% Tween 80 was used as negative control and Tween impregnated disc hasn’t showed any inhibition against the test organism which implies that the inhibition observed was exclusively by the crude extracts (Table 6).
DISCUSSION
In this study the antimicrobial susceptibility test was conducted on two species of bacteria namely \textit{S. aureus} and \textit{E. coli} isolated from bovine mastitis and two medicinal plants namely serially extracted \textit{C. molle} seed with four different alcohols and FR1 leaf extracted by Ethanol and methanol. Commercially available antibiotic discs (Ampicilin) were used to compare 200mg/ml (4mg/disc) extraction of these medicinal plants. The result indicated that \textit{S. aureus} and \textit{E.coli} were susceptible to \textit{C. molle} seed and FR1 leaf compare to positive controlled (Ampicilin).

The Tween 80 used as the solvent to make different concentrations was used as a negative control and there was no inhibitory effect and the result obtained from this study was purely related to the efficacy of each type of alcoholic extracts of the phytopreparation.

When zone of inhibition of each absolute methanol extract of FR1 at different concentration compared with its ethanol extract, it showed better inhibitory effect on test organisms. Mean Zone of inhibition of ethanol and methanol soluble fractions of different concentration of \textit{C.molle} seed was found better than ethanol and methanol extracts of FR1 leaf against \textit{S. aureus} and \textit{E.coli}.

\textit{C. molle} seed had been studied for antimicrobial effect on \textit{S. aureus} and \textit{E.coli} while there was no work done on different solvent. The acetone soluble fraction of \textit{C. molle} seed showed a wider inhibition zone against \textit{S. aureus} than other fractions. Ethanol soluble fraction of \textit{C. molle} seed has wider zone of inhibition on \textit{E.coli} than other alcoholic fractions. Minimum zone of inhibition at lower concentration is observed on methanol soluble fraction of \textit{C. molle} against \textit{S. aureus}. From this simple observation it is possible to hypothesize that different ingredients of the plant/herb have different solubility depending on the solvent used. Hence, the type of solvents used matter for the efficacy of the plant/herb.

However, FR1 has negative effect at its lower concentration against \textit{E.coli}, its methanol extract has equivalent zone of inhibition with that of methanol soluble fraction of \textit{C. molle} at higher concentration against \textit{S. aureus}. whereas \textit{C. molle} showed antibacterial effect at all concentration on both test organisms. In this regards, all alcoholic fractions of \textit{C. molle} seed has shown better activity at its lower concentrations which disagreed with previous work by Regassa and Mengistu. (2012).

In this study a direct relationship between concentration and zone of inhibition was observed. Therefore, in all case of the test plants with antimicrobial activity, there was dose dependent inhibition on the tested bacteria showing greatest activity at highest concentrations of the crude extracts which agrees with previous works by Mengistu (2004), Sahlu (2013), Kenide (2014) and Taddese (2007) even if there is a difference in zone of inhibition at different concentration levels.

Comparison of herbal preparations with conventional antimicrobial discs was made only by the size of zone of inhibition obtained by each test materials against test organisms. The mean inhibition zone obtained by Ampicilin against isolates of \textit{S.aureus} was found lower than all extracts of both plants at higher concentration. This result may indicate that the bacteria are resistance to Ampicilin. Antibacterial activities of both 200mg/ml methanol extract of FR1 and 200mg/ml of acetone soluble fraction of \textit{C. molle} against \textit{S. aureus} the activity of the standard antibiotic (Ampicilin) with mean zone of inhibition 16.33 and 18.33 respectively. Also 200mg/ml ethanol soluble fraction of \textit{C.molle} shows comparable antibacterial activity against \textit{E.coli} with that of the standard antibiotic (Ampicilin) with mean zone of inhibition 19.33. The comparison among these test materials suggests that the herbal preparations do have a capacity to inhibit the growth of test organisms with a similar or a different manner to that of
conventional antimicrobial agents, though there are no established standard formulae to judge the level of zone of inhibition to say resistant, intermediate and susceptible for Phytopreparations.

In general, the variation of efficacy among the above-mentioned phytopreparation against the test organisms could be attributed to the way of plant preparation, season of collection, stage of the plant, place of collection, way of extract drying, means of extraction, solvent used, preservation or storage of the extract till evaporation, amount of bacteria swab streaked on the agar plates containing Muller-Hinton agar and other unnoticed factors.

**Photograph of plants used for the test**

![Figure 4. Combertum molle seed.](image)

![Figure 5. FR1 leaf.](image)

![Figure 6. FR1 leaf macerated in methanol extracted in rotavapor Crude extract](image)
CONCLUSION AND RECOMMENDATIONS

As antibiotic use increases in veterinary medicine, the issue of bacterial resistance to antimicrobial therapy becomes more worrisome. Losses due to clinical mastitis cases are frustrating the dairy industry and resistance of the bacteria which causes mastitis for commonly used antibiotic is increasing from day to day. Also wide spread use of antibiotic for the treatment of bovine mastitis has a potential to cause contamination of milk, which has become a subject of public concern. Therefore, one way to control drug resistance and alleviate the problem is through the development of alternative antimicrobials from natural resources (herbs/plants) by screening and testing medicinal plants for their possible antimicrobial effects. The present study was conducted on different alcoholic extracts of selected two medicinal plants and the results found against two bacterial species (S. aureus and E. coli) which were isolated from mastitic cases were very encouraging. The results found in this study are indicatives that these plants can be used as a source for the isolation of active compounds that may serve as leading compounds in antibacterial drug development and be relief for the increasing problem of antibiotic resistance. From this point of view plants/ herbs...
that are documented to have effects on the treatment of mastitis and other diseases will have to be tested against a range of existing conventional medicament and validated. Therefore, from the finding of this study the following recommendations were forwarded:

- Further studies on Phyto-chemical analyses should be conducted on these plants and the active chemical should be identified.
- In-vivo efficacy and safety studies are required to evaluate the therapeutic value of the plants against bovine mastitis.

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REFERENCES


Mungube, E.O. (2001). Management and economics of dairy cow mastitis in the urban and peri-urban areas of Addis Ababa MSc thesis free University of Berlin and AAU.


Sahlu, T. (2013). Antibacterial activities and preliminary phytochemical investigation of four selected medicinal plants namely leaves, steam, bark and seeds of *C. molle*, steam bark of *Bereza* and leaves of *Xanthium strumarium* and *Laggoria arota* against *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli*. (UN published DVM thesis, AAU).


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