



Phytochemical Analysis and Antibacterial Activity of Crude Extract from *Eucalyptus globulus* Leaves Against Selected Bacterial Pathogens

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Abstract: Extracts from *E.globulus* that have phytochemical and antibacterial properties against pathogenic microbes such *S. aureus*, *P. aeruginosa*, *E. coli*, and *S. pyogenes*. The objective of this work was to ascertain the lowest inhibitory concentration of ethanol and aqueous leaf extracts on *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. pyogenes*, as well as to analyze the phytochemical and antibacterial activities of *E.globulus* extracts. After being gathered from the Hosanna area, *E.globulus* leaves were taken to the Wachemo University Biotechnology lab for additional processing. Plant extracts were weighed (100gm) for antibacterial activity and phytochemical analysis, and serial dilution was used to calculate the minimum inhibitory concentration (MIC). The SPSS program was used to do the analysis of variance. At a significance threshold of $p < 0.05$, treatment means were compared. The presence of phytochemicals was demonstrated by a qualitative phytochemical screening test of ethanol and aqueous crude extract. The ethanol leaf extract demonstrated a greater zone of inhibition against *P. aeruginosa* (25.900 ± 0.1 mm), while the aqueous leaf extract demonstrated a significant zone of inhibition against *S. pyogenes* (21.4 ± 0.1 mm). In order to develop new antimicrobial medicines from *E. globulus* plants, more study into the separation of bioactive compounds is therefore feasible.

Keywords: *phytochemical, alkaloids, flavonoids, antimicrobials, antibacterials, and saponins*

1. Introduction

1.1 Background Of The Study

Around the world, people employ medicinal plants as complementary treatments for both physical and mental illnesses. The majority of people in rural areas believe that traditional health practices and herbal compound remedies are more affordable and easily available than contemporary medications (Jima and Megersa, 2018). The World Health Organization (WHO) estimates that approximately 65% of rural residents receive primary health care from medicinal plants. Additionally, since 1980, nearly 39% of pharmaceuticals have been derived from plants (Johnson et al., 2020). Traditional medical practices and therapies can be traced

back to early medico-spiritual texts, oral traditions, and 40th-century conventional pharmacopoeias (Vecchio et al., 2016). Many *in vitro* studies have examined the effectiveness and safety of the Ethiopian medicinal plant, which is used to treat bacterial and fungal illnesses. Ethiopian medicinal plants are plants found in Ethiopia that have long been utilized for therapeutic purposes. For *in vitro* anti-infective purposes, bacteria are directly subcultured into media, and plant extracts are applied to the culture media to ascertain their activity. Anti-infectives are active components (compounds, extracts, and/or fractions of medicinal plants) that bind to infectious pathogens by blocking

and/or inhibiting their proliferation. In the majority of rural locations, plant debris prevents the of infectious infections, wounds, and inflammation (Gebremeskelet al., 2018).Australia and Tasmania are the native home of the big evergreen eucalyptus plant. Among the more than 900 variations and subspecies in the Myrtaceae family, it is a member(Wacquantetal., 2014). Antiseptic, antioxidant, anti-inflammatory, and anti-cancer effects have been found in the bark, buds, fruits, and leaves of the plant. It helps avoid a lot of respiratory illnesses based on these activities. like the flu, sinusitis, constipation, and the common cold (Patricia Gull et al., 2020).manufactured via microbial biosynthesis, however others are synthesized.

Antimicrobial agents are substances that intervene in microbial a metabolism. These substances are produced within the chemical laboratory or obtained from living organisms. They are naturally occurring substances which called antibiotics. Oil extracted from *E.globulus* with slightly diluted glycerin can absolutely treat facial demodicidal (Kesharwaniet al., 2018).

Today, *E. globulus* is widely cultivated worldwide (Joshi et al., 2016)due to its easy adaptability to environmental conditions, ease

of cultivation, rapid growth rate, and increase in woody biomass. Also, it can be planted in contaminated regions (Amano et al., 2021).

2. Materials and Methods

2.1. Description of study area

Hosanna is also known as Hosa'ina, Hosaena, Hosana, Hosanna, Hosa'ina, Hossana, Hosseina, Hosäenā Located in the Southern Nations, Nationalities, and People's Region (SNNPR), Hossana has a latitude and longitude of 7°33'N 37°51'E with an elevation of 2,279 meters above sea level.For administrative purposes, the zone is structured into 10 districts and a Town Administration.Hadiya Zone is located 232 km South West of Addis Ababa, the capital of Ethiopiavia Alemegena-Butajira route, 280km from via Wolkite route, and 305km via Ziway. Hossana is located in southeast of Hawassa the capital of Sidama at approximately 168km via Halaba-Angeca and 203km via Halaba. Hossana also located 97km north of WolyitaSodo town and 76km North of Durame town. The town is also bounded by peasant associations namely, Ambicho and kalisha in the North, Lareba, Jewic and Haysicin the South; Ambicho in the east; and Gora, Bobicho and Allela in the West (United Nation Development Program (2000).

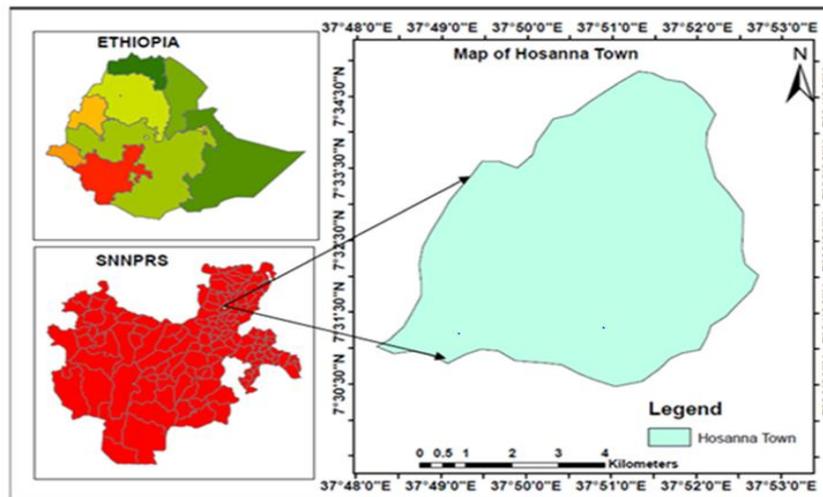


Figure2:Map of Hosanna Town

2.2. Plant sample collection

The plants were randomly collected in densely populated areas from around Hosanna town. Fully fresh *E. globulus* leaves were gathered. These samples were transported to Wachemo University's Department of Biotechnology on February 2022 after being packaged in cotton bags. The plants leave with no dead leaves; flowers and different height were selected and placed on a table. The samples were washed by tap water to remove dirty and contaminants. Leaf samples from *E. globulus* plants were air-dried for two weeks at 25°C in the Biotechnology Laboratory. They were then put through an electric grinder to create a coarse powder. The obtained dry powders were kept in airtight plastic containers and kept chilled at 4°C.

2.3. Preparation of plant extract

A 100gm of the powdered sample from fresh leaves of the *E. globulus* was weighed and placed in a bottle. Then a volume of 500 mL of ethanol was added in bottle and sealed. The mixture was left for 72 hrs. After 72 hrs the filtration of the extracts was then done using Whatman # filter papers No.1. The solvent was filtered and then evaporated under reduced pressure using a water bath. Filtration processes and extracted plant leave showed in figure (2 and 3)



Figure 3: Ethanol and water extraction processes



Figure 4: leave extracted crude

This entire extraction processes gave a viscous dark brown residue of water extract and a viscous dark green residue of ethanol extract. The extracts were then placed in open 100 mL beakers for 5 days to allow any remaining organic solvent to evaporate until a sticky solid was formed, which was then stored at -4°C until the experiments began.

2.4. Phytochemical analysis for *E. globulus*

2.4.1. Qualitative phytochemical analysis

The coarse powder of *E. globulus* leaves and crude extracts had a phytochemical analysis perform for the existence of bioactive compound via normal techniques (Jamil et al., 2017). The 0.2gm of crude extract was weighed and dissolved in a volume of 5ml dimethyl sulphoxide (DMSO) for phytochemical analysis.

2.4.2. Testing for flavonoids

Shinoda test: Two sterilized test tube were placed in a test tube rack and 2 ml of crude extract solution was poured into the test tube and labeled A and B. Three fragments of magnesium ribbon and 2ml of concentrated hydrochloric acid was added drop wise in

second test tube (B). The result of this was given in Table (6).

Alkaline reagent test: A 2 sterilized test tube were placed in a test tube rack and 2 ml of crude extract solution was poured into the test tube labeled A and B. Two drops of sodium hydroxide solution were added in second test tube (B). An intense yellow color formed. In the second test tube (B) was also added two drops of dilute HCl acid and observed.

2.4.3. Testing of saponins:

A 2ml of Crude extract was added using dropper into test tube. Equal amount of crude extract in second test tube which used as control. Then after transferring 2ml of crude extract solution in both test tube and 5ml of distilled water was mixed in a second test tube Crude as control. The solution was shaken vigorously and observed the result it showed in table (6). If honeycomb froth greater than 2 cm from the surface of the liquid persists after 10 minutes, the sample is considered positive for saponins.

2.4.4. Testing of alkaloid

Wagner's test: Two sterilized test tube were placed in a test tube rack and 2 ml of crude extract as solution was poured into the test tube and labeled A and B. crude extract was used as control. A 1 ml of Wagner's reagent (iodine and potassium) which used as positive control iodide was added drop wise. The color change was recorded table (6).

Mayer's test: 2ml of Crude extract solution was poured into test tube to the help of dropper and transfer equal amount of crude extract solution in second test tube. 1 ml of Mayer's reagent were added in second test tube in drop wise. Potassium mercuric iodide solution as positive control. The formation of color change the result was showed in table (6).

2.5. Antibacterial Activity

2.5.1. Test bacterial organisms

The tested microorganisms used in this study were included, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Pseudomonas aeruginosa*.

2.5.2. Collection of human bacterial test

The microorganisms were collected from Ethiopian Public Health Institute (EPHI) Addis Ababa, Ethiopia with the cooperation of the laboratory team. Each of these four bacterial strains was found in the plate. *E. coli* (ATCC25922) peptone water and labeled as S1, *S. aureus* (ATCC25923) as S2, *S. pyogenes* (ATCC28422) as S3 and *P. aeruginosa* (ATCC27853) as S4. All these collected bacteria were placed in Stuart's and transported from EPHI to Wachemo University Biotechnology Lab.

The transport media used was Stuart's with a relatively types of swab. Composition of Stuart's: -sodium glycerophosphate (10.00gm/lit), sodium thioglycollate (1.00gm/lit), and CaCl_2 . They were kept as stock cultures in a refrigerator at a temperature between 4°C. The cultures of the clinical isolates of the test microorganisms were maintained on agar slants.

The nutrient agar slants were prepared by dissolving 28gm nutrient agar on 1000ml of distilled water. The bacterial microorganisms were streaked on nutrient agar slant. The streaked agar slants were placed in an incubator at 37 °C and observed for growth after every 24 h. The test microorganism was subsequently sub cultured after every 48h to maintain their viability. From the each pure culture some colony transferred in four different test tubes which contains nutrient broth and incubated 37 °C for 24h.

2.5.1. Preparation of nutrient agar and nutrient broth

Nutrient agar is a general purpose, nutrient medium used for the growth cultivation of microbes supporting growth of a wide range of non-fastidious organisms and basically, the

nutrient broth is the nutrient agar that lack of the solidifying agent, agar powder. The remain in liquid form at room temperature and are usually used to maintain the stocks of microorganisms. In general, they are used to grow fastidious organisms. The composition of nutrient agar and nutrient broth Beef extract 3.0 gm, Peptone 5.0 gm, Sodium chloride 8.0 gm, Agar (15.0 gm), Distilled water (1000ml), PH (6.8) and Beef extract 3.0 gm, Peptone 5.0 gm, Sodium chloride 8.0 gm, Distilled water (1000ml), PH (6.8) respectively. These growth media were prepared using the standard protocol, Monica (2000). A 28gm of the nutrient agar powder was dissolved in 1000 ml of distilled water in conical flask and autoclaved at 121°C for 15 min and then allowed to cool to 25°C and dispersed in plates. The plates were used for culturing and susceptibility testing of the organisms.

2.6 Antimicrobial susceptibility test

antibacterial activity. A second stock solution of the extract (200 mg/ml) was made by reconstituting 200 mg of each of the dried extracts in 1 mL of ethanol. Serial two-fold dilutions were then made from the stock solution to create the various concentration solutions as follows: Five test tubes were sterile, set on a test tube rack, and filled with nine milliliters of nutritional broth each. The extract was serially diluted one at a time after transferring 1 milliliter of the stock solution to the first test tube. The resulting concentrations in the test tubes were 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, and 6.25mg/ml (Esimone et al., 2012). A 0.1ml of standardized inoculum of overnight broth culture was inoculated into the dilutions and incubated at 37°C for 24h. The MIC was taken as the least concentration that inhibited the growth of the test Organism.

Following cooling, the discs were impregnated using micropipettes to apply a stock solution containing 200 mg/ml of the

prepared test solutions for ethanol and water extracts. This was accomplished by putting the stock solutions and sterile discs in a laminar flow. disc was kept in the stock solution of plant leaf extracts for one hour. After that, the discs were taken out and put in a sterile Petri dish with a laminar flow to dry for fifteen minutes. After being plucked with sterile forceps, the impregnated discs containing leaf extracts were placed in a sterile Petri dish and kept in a refrigerator at a temperature of at 4–8 °C prior to being utilized in the test for antimicrobial susceptibility (Taura et al., 2014). Using the Kirby-Bauer method, the antimicrobial efficacy test was conducted (Newallet et al., 1996). The spread plate technique, which involved spreading the clinical isolates using sterile cotton wool swabs, and the bacterial test both employed nutrient agar.

While rotating the nutrient agar plates by around 60° each time to ensure a uniform distribution of the inoculum.

In order to allow any extra surface moisture to be absorbed, the plates were then left open for 3-5 min (CLSI, 2012). They were exposed to extracts impregnated discs in milligrams per micro liter from *E.globulus* leave extract. The discs were placed with equal distance between them on agar plates inoculated with the bacterial pathogens

Following this the extract solution were pipetted to the discs the impregnated discs were dispensed onto the surface of the inoculated agar plate using sterile forceps used three paper disks for one plate. Each disc was pressed down to ensure complete contact with the agar surface. The discs were equally spaced so that they were 24 mm or more apart from center to center (CLSI, 2012). Commercial Gentamycine discs (5µg/ml) served as the positive control was used for the all four bacteria's and discs impregnated with pure solvent ethanol (20 µL and 30 µL)

and water (20 μ L and 30 μ L) served as the negative control.

Each time, a sterilized forceps was used to carefully place two prepared discs containing the various extracts and one antibiotic disc on the ten (3) inoculation plates. The plates were then upside-down and inoculated in an incubator for 24 h at 37°C.

Following incubation, the diameters of the inhibition zone surrounding each disk were measured side by side. A clear ruler was used to measure millimeters along two axes that were 90° apart, and the mean of the two readings was recorded. The experiment was carried in duplicates and the diameter of zones of inhibition formed was measured and their average determined. (Thompson et al., 2011 and Biswas et al., 2013)

2.7. Minimum Inhibitory Concentration Determination Antibacterial testing.

The minimum inhibitory concentrations (MIC) of the absolute (stock) concentrations were determined using the serial dilution method; five sterile test tubes were prepared and 9ml nutrient broth was poured into each of the test tubes and 0.1 ml of the prepared concentration of extract was mixed with the nutrient broth. Thereafter, standardized inoculum of 0.1 ml of the test pathogen was dispensed into the test tube containing the suspension of nutrient broth and the extract. Then, all test tubes were properly corked and incubated at 37°C for 24 hrs. The activity was observed as negative if growth was there and positive if the medium appeared clear without any growth of the microbes and the minimum inhibitory concentration was.

Thereafter, they were examined for the absence or presence of visible growth. The lowest concentration with no visible growth of organisms was taken as the MIC. The experiment was performed in duplicate for each organism (Taura et al., 2012).

2.9. Data Management and Analysis Method

The data collected was exported to Microsoft excel spreadsheet where descriptive statistics were carried out. Statistical Package for Social Science, Version 20 (SPSS) is used to analyze the data. The recorded from antibacterial disc diffusion tests, are analyzed using a one-way analysis of variance (ANOVA). The significance of the statistical tool output was set at $p < 0.05$. Means were separated using Duncan's New Multiple Range Test (DNMRT) at 5% level of probability using Statistical Analysis Software (SAS) package

3. Discussion

Phytochemical screenings of extracts were varied between plants of the same species. That is, it may vary from place to place depending on geographic location, climatic conditions, and habitat soil composition. This may help explain why it makes sense for the plants we tested to have differences in chemical composition compared to different plants tested in other regions. The plant contains active ingredients that are effective in preventing microbial infection, as shown by disc diffusion assay results. Moreover, some species, such as antimalarial and influenza, interact with bacteria very actively (Gupta et al., 2012). Qualitative analysis results indicated the presence of alkaloids, saponins and flavonoids in leaves. Similar to the current study (Taura et al., 2014), alkaloids, flavonoids and saponins were found. In addition, it exhibited an antibacterial effect. Nutrient agar was used for bacterial test and in the spread plate technique where the clinical isolates were spread using sterilized cotton wool swabs.. The plants used in the study are said to have medicinal properties. This study evaluated the use of botanicals in the treatment of selected bacterial pathogens (*E.coli*, *P.aeruginosa*, *S.aureus*, and *S. pyogenes*) and tested whether it was effective when used alone. The results of this study show that crude extracts of *E.globulus* (aqueous and ethanolic) act to

inhibit the growth of selected pathogenic bacteria and are less than the zone of inhibition of the positive control gentamicin (5 µg/disc). However, *S. aureus* has the smallest average diameter zone of inhibition at the concentration of plant extract (20 µL). Other bacterial tested at the same concentration (20 µl) had a slight effect. The average zone of inhibition of plant extracts at his 30 µl concentration for all bacteria tested was greater than the lowest plant extract (20µl) and not significantly different ($p < 0.05$).

Antimicrobial screening results for zones of inhibition of study eucalyptus plant extracts against each bacterium tested were inversely proportional to their MIC values. These oils can be extracted from eucalyptus leaves and used in incredibly small doses for antiseptic, purifying, and disinfectant properties as well as in food additives, primarily for desserts, decongestants, and cough drops (Igbe Iet al., 2010). Infectious diseases are the leading cause of death worldwide, accounting for almost half of all deaths in tropical countries, and are becoming a major problem in all countries. Microbial infections pose major challenges to human health, exacerbated by increased resistance to conventional drugs (Ibezim, 2005). Therefore, researchers need to find plant-based remedies against infectious diseases. The leaf extract showed a moderate inhibitory effect against the selected bacterial pathogens at 20 and 30 µl concentrations of test extracts. In this study *P. aeruginosa* was found to be the most susceptible to crude ethanolic extracts that obtained from *E.globulus* with of zone of inhibition (25.9 ± 0.1 mm). Aqueous leaf extract was also shown the least antibacterial activity against *S. aureus*, with zone of inhibition (16.4 ± 0.1 mm), and Furthermore aqueous leaf extract also showed the highest antibacterial activity against *S.pyogenes* with considerable zone of inhibition (21.4 ± 0.1 mm).

The crude extract and pure components are known to be active against a wide range of

bacteria, including Gram negative and Gram positive bacteria. Therefore, leaves of *E.globulus* extracts are valuable and could be a future target for replacing synthetic antibacterial agents. The antibacterial activities of the tested plant leaf extract were more pronounced on the Gram-negative bacterium (*E. coli* and *P. aeruginosa*) than on the Gram-positive bacterium (*S. aureus* and *S. pyrogenes*).

Gram-positive cell walls are made up of many peptidoglycan layers and have no outer lipid membrane. Gram-negative cell walls, on the other hand, have only one or a few layers of peptidoglycan, but have an outer membrane composed of various lipid complexes. Furthermore, while gram-positive spores develop as a new structure within the protective interior of an existing cell wall, Gram-negative cells directly transform into spores while maintaining the structural integrity of the cell wall (Liu et al., 2004)

The differences between Gram-positive and Gram-negative organisms only demonstrate that the passage of certain small molecules (inorganic phosphate, nucleotides, etc.) through the wall or wall membrane of gram-positive bacteria is impeded when they are suspended in high concentrations of the sample, while many of the gram-negative bacteria are less so extent were affected (Salton, 1951).

6. Conclusion

In this result flavonoids, saponins and alkaloids were detected in this plant leaf extract. The plant extracts showed antibacterial against *S.aureus*, *E.coli*, *S. pyogenes*, and *P.aeruginosa* showed good effects, which promotes the use of this plant as a medicine against various types of diseases.

These results also confirm that the potent use of *E.globulus* in the curative pharmaceutical industry could be beneficial as an alternative

antimicrobial agent in homegrown drugs to treat multiple diseases. This study confirmed that ethanol and aqueous leaf extracts of *E.globulus* were able to inhibit the growth of test organisms. The activity of plant materials tested had varied inhibition zone which were the dependent extract solvent. Although ethanol extracts had the best activity, than aqueous extract at different concentration.

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