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Efficacy of ethanolic extract of *chewing sticks of Olea europaea* sub spp. cuspidata against bacterial isolates from human oral flora

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ABSTRACT

The oral cavity of humans is a habitat for gram positive and gram negative bacteria, as well as certain other yeasts and fungi making it one of the most complex microbial habitats in the body. Due to different types of microorganisms that are highly affecting oral cavity, a large number of people are engaged in a problem and they are affected with halitosis and other diseases, but there is no scientifically known studies about the traditional chewing sticks of O.europaea against oral health. The main purpose of this study would focuses on the efficacy of Ethanolic extracts of O.europaea against bacterial isolated from oral flora of human beings. The bacterial samples were isolated from human oral cavity and identified by staining and biochemical methods; then prepared Ethanolic Crude extracts of O.europaea sticks and tested their efficacy against bacterial isolates using disc diffusion method. The studies suggested that Ethanol extract of sticks of O.europaea were inhibited the growth of the test bacterial isolates obtained from the human oral cavity. Usages of chewing sticks are gradually disappearing to modern teeth cleansing materials, oral hygienic chemicals and non- availability of chewing sticks. Further phytochemical analysis on O.europaea sticks will lead to provide promising lead molecules which might be useful to prepare indigenous oral hygienic/ cleansing agent(s).

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1. Introduction

The oral cavity of humans is a habitat for Gram positive and Gram negative bacteria, as well as certain yeasts and fungi, making it one of the most complex microbial habitats in the body. Although saliva contains lysozyme and lacto peroxidase, which are both anti bacterial agents, the presence of food particles and shaded epithelia cells, makes the oral cavity of a favorable microbial habitat at $37o_c$ with neutral P^H (Madigan M.*Jetal*.,2003.). Dental caries is break down of teeth due to the activities of bacteria. Symptoms may include pain and difficulty with eating, complications may include inflammation of the tissue around the tooth, tooth loss and infection or abscess formation (Silk, 2014).

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The tongue is modification of the squamous epithelium lines the oral cavity. Saliva is composed on several functional components which aid in lubrication, enamel, demineralization, digestion, and aggregation and provides oral buttering (Devine, D.A *et al*., 2008).

Oral communal bacteria are important as they can regulate the expression of immune mediators, suppress cytokine response in epithelial cells and prevent colonization by exogenous organisms such as *Streptococcus remites, Streptococcus orals, Antinomies naeundiis, Fusabacterium nucleatum, Homophiles pasras influenza* and certain privately species may be oral commensals as they are isolated from healthy but not diseased sites (Devine,D.A *et al*., 2008).

Worldwide, approximately 2.43 billion people (36%) of the population have dental caries in their permanent teeth. The WHO estimates that nearly all adults have dental caries of some point in time in body teeth. It affects about 620 million people or 9% of the population. They have become more common in both children and adults in recent years. The disease is most common in the developed world and less common in the developing world due to consumption of greater simple sugar foods (Bagromion, R *et al*., 2009).

Due to different types of bacteria that are highly affecting oral cavity, a large number of people are engaged in a problem and they are affected with halitosis and other diseases, but there is no scientifically known studies about the traditional chewing stick (*Olea europaea*) against these disease.

This project is mainly aimed to evaluate the efficacy of traditional chewing stick *(O. europea*) against the bacterial isolates from human oral flora (students of Aksum University) in order to reduce the problems facing by the microbes found in the oral cavity.

This verifies that there could be several reasons for this situation including the deficiencies in health system that leads to lack of access to teeth problems control interventions and low effectiveness interventions than expected. Even if people uses different types of chewing stick *(O.europaea)* to remove food colonies in their mouth, oral bacteria are affecting a lot of peoples in Ethiopia. This is because improper use of chewing sticks and people lack of awareness about antibacterial activity of Olive *(O.europaea*).Thus it is very essential to conduct research project in this direction to identify the gaps and solve the issues or problems certain extent. Therefore this work involves community approach to confirm the efficacy of traditional chewing sticks (*Olea europaea*) against bacterial isolates obtained from in oral cavity of human beings (Students of Auk).

2. Materials and Methods

2.1 Description of the study area

The study was conducted in Tigrai Regional state, central zone of Tigrai, at Aksum University, Department of Biotechnology (in Microbiology laboratory).

2.2 Study design

This study was experimentally using the appropriate methods such as isolation, identification of bacteria with the help of microscopic examination, biochemical test and antibiotic sensitivity test using disc diffusion method.

2.3 Data collection

Data was collected by using primary data collection method. The sample would be collected from the trees of olive around Axum town and bacteria were isolated from oral flora of human beings (students of AkU).

2.4 Materials

Aluminum foil, Autoclave, Beaker, Coffee grinder, Conical flask, Cover slip, Filter paper, Flame, Forceps, Hot plate, Incubator, Inoculation needle, Magnetic stirrer, Measuring cylinder, Microscope, Paper disc, Petri dish, Refrigerator, Separator, Slip, Spatula, Stirrer, Swab, Test tube and Inoculation loop,

2.5 Chemicals

Ampicillin, distilled water, Ethanol, Ethanolic extract of *O. europaea*, NAM and sterilized water

2.6 Laboratory procedure

Laboratory procedures such as sample collection, sample processing, bacterial culture, microscopic examination, biochemical tests and antibiotic sensitivity test were used to determine the efficacy of ethanolic extracts, isolation and identification of bacteria obtained from human oral flora.

2.7 Sources of the sample

The traditional chewing stick (*O. europaea*) is easily found around Axum town and the bacteria were isolated from oral flora of human beings (students of AkU).

2.8 Collection of sample

The sample of bacteria from oral flora of humans (students of AkU) was collected from march 28, may 10, and the sticks of Olive*(O.europaea*) was collected from the trees of Olive *(O.europaea*) around Axum town from march 18-23 may.

2.9 Preparation of samples

2.9.1 Sample preparation of bacteria form oral flora of humans

The bacterial sample was collected from human oral flora (students of AkU). The bacterial isolates were deposited at the department of Biotechnology laboratory for further processing such as isolation, identification and antibiotic sensitivity test.

2.9.2 Sample preparation for *Olea europaea* crude extract

The sticks of olive (*O. europaea*) were collected from the trees found in around Axum town and the sticks of olive *(O. europaea)*were deposited to the department of biotechnology laboratory to shade dried and milled to coarse powder then subjected for solvent extract. The plant material was extracted with organic solvent ethanol (97%) for 3 days or until the plant material was discolored. The extracts were filtered and evaporated. The solvent extract thus obtained was tested for antibacterial activity using anti bacterial sensitivity test.

2.10 Media preparation

2.8g of NAM was weighed using a clean electronic weighing balance; 100ml of sterile distilled water was poured in to a conical flask. The mixture was shaking by using hand and covered with wool. Over which an aluminum foil was tightly warped and then heated for few minutes using hot plate until the foam formed. And the medium together with Petri plates were sterilized in autoclave for 15 minutes at 120^0 c. Soon after autoclaveing, the agar was allowed to cool and placed inside a water bath at about 50^0 c to maintain the media in a molten state (to minimize the amount of condensation that forms). Then the agar medium was allowed to cool to room temperature prior to pouring it into the Petri plate. Plates were dried faster in lower humidity by keeping them at room temperature. The freshly prepared and cooled medium was poured into flat bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximate 4 mm. This was achieved by pouring 20 ml of the medium for plates with diameters of 100 mm. then; samples were separately cultured in nutrient agar at 37^0c for 24 hrs by streak plate method.

2.11 Bacterial identification

2.11.1 Gram staining procedure

The slide was placed with heat fixed smear on staining tray. A drop of crystal violate was added and allowed for 60 seconds and then washed with distilled water and also drop of Iodine was added and then allowed for 30 seconds after this washed by distilled water. Then 95% ethyl alcohol (decolonization) was added and allowed for 30 second then washed by distilled water. After that safranine was added and allowed for 60 seconds and then washed with distilled water finally the slide was put under microscope and then the purple color and pink color from a single colony of the slide under microscope was observed. Then the purple color from a single colony of the slide under microscope indicated that the bacteria were gram positive were as the pink color observed under microscope that indicates the bacteria were gram negative. Microscopic investigation for staining method and morphologic features of isolated bacterial colonies were determined using standard method of Grams staining.

2.11.2 Biochemical test

Relevant biochemical test were carried out to aid in the identification of the bacteria in partial manner. Catalase test and motility test (Stabbing method) were used, describes by Barrow and Fathom (1993).

2.11.3 Antibiotic Sensitivity test using disc diffusion method

The antibacterial activity of selected chewing sticks of *O.europaea* was tested against bacterial isolates from human oral flora through disc diffusion method. The dry extract was initially dissolved in ethanol and then

1. Place a sterile Swab into the broth culture of specific organism and gently remove the excess liquid by gently pressing or rotating the Swab against the inside of the tube.
2. Using the Swab, Streak the NAM plate to form a bacteria's lawn; To obtain uniform growth, Streak the plate with Swab in one direction, rotate the plate $90^0$and Streak the plate again in that direction - Repeat this rotation 3 times
3. Allow the plate to dry for approximately 5 minutes.
4. Use antibiotic Disc dispenser to dispense discs containing specific antibiotics on the plate.
5. Using flame sterilized forceps, gently press each disc to the agar to ensure that the disc is attached to the agar. Finally allow for incubation and after 24 hours or 48 hours measure zone of inhibition

2.12 Data Analysis

After the data had been collected properly the researchers have organized and summarized the collected data. The data was analyzed carefully through both quantitative and qualitative methods of data analysis techniques.

Qualitatively through microscopic observation of isolated samples, biochemical tests and quantitatively through measuring of efficacy of *O. europaea* crude extract on the bacterial isolates from oral flora of human beings using disc diffusion.

3. Results and Discussion

3.1 Results

Table 1 Antibacterial spectrum of Ethanolic extracts of *O.europaea*

S.No.	Test Sample (g/L)	Area of Inhibition zone(mm^2)			
		A^	B^	C^	Average - (A+B+C) / 3
1	*Negative Control (0.5 ml)	6	6	6	6
2	Ethanolic plant Extract (12.5/75)	15	12	13.5	13.5

^A, B and C replicates of the test sample

* Negative control - Ethanol 97%

Figure 1 Ethanolic Plant Extract-Inhibition Zone

Table 2 Antibacterial Spectrum of Negative control and positive control (Std)

S.No.	Test Sample (g/L)	Area of Inhibition zone(mm^2)			
		A^	B^	C^	Average – (A+B+C) / 3
1	*Negative Control (0.5 ml)	6	6	6	6
2	@Positive control (1g/9mL)	14	10	13	12.5

^A, B and C replicates of the test sample

* Negative control - Ethanol 97%

@Positive control (Ampicilin 10%)

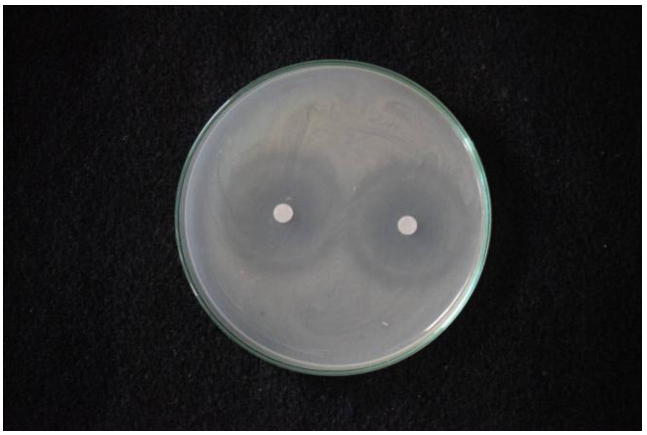


Figure 2 Zone of inhibition –Standard (Ampicillin)

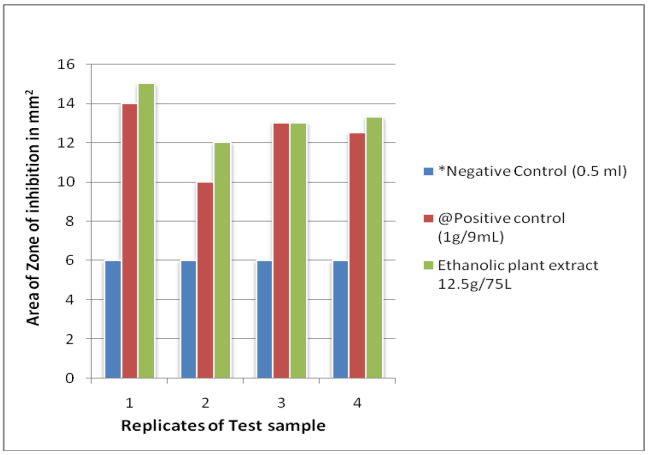
Table 3 Antibacterial spectrum of ethanolic extracts of *O.europaea* and standard (Ampicillin)

S.No.	Test Sample (g/L)	Area of Inhibition zone(mm^2)			
		A^	B^	C^	Average - (A+B+C) / 3
1	*Negative Control (0.5 ml)	6	6	6	6
2	@Positive control (1g/9mL)	14	10	13	12.5

3	Ethanolic plant extract 12.5g/75L	15	12	13	13.3

^A, B and C replicates of the test sample
* Negative control - Ethanol 97%
@Positive control (Ampicilin 10%)

Graph1 Graphical representation of Antibacterial spectrum of ethanolic extracts of sticks of *O. europeae*



1, 2 and 3 are replicates of the test sample
4 indicates Average zone of inhibition of the test sample

Table 4 Gram Staining test

S.No	Gram stain test	Characteristic Features	Results
1	Bacterial sample 1	Pink color ,spiral and rod shaped	Gram –ve, spirals and rods
2	Bacterial sample 2	Pink color ,spiral and rod shaped	Gram –ve, spirals and rods
3	Bacterial sample 3	Purple and Pink color ,ring and rod shaped	Gram –ve, gram +ve spirals and rods

In a smear that has been stained using Gram Stain protocol, the Shape, arrangement and gram reaction of bacterial culture was revealed. The observed result under microscope in test sample 1 and 2 was pink (red) where as test sample 3 was in the half part purple and in the half part pink, so the bacteria in sample 1 and 2 were gram negative and the bacteria in test 3 was mixtures of both gram negative and gram positive.

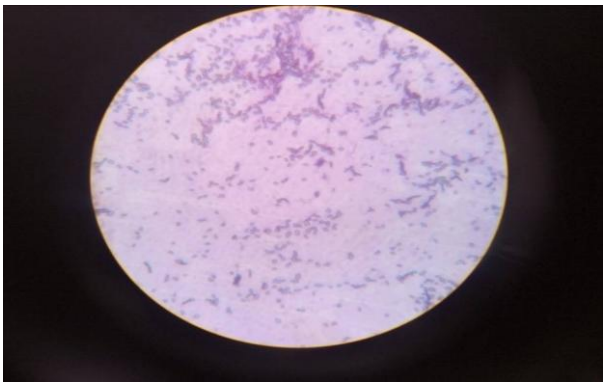


Figure 3 Microscopic photograph of gram negative rod shape and spiral shaped bacteria

Table 5 Biochemical test

S.No.	Test	Observation	Results
1	Catalase	Bubbles were formed after adding H_2O_2	+Ve
2	Motility test	The sample was diffused around the stab line in agar tube	Motile

The observed result using Catalase test was while adding H_2O_2; there was bubble formation; this indicates that the sample is catalase positive. Also in motility test while caring out using stabbing technique the sample in the Agar tube/ Agar deep using inoculation needle, growth around the stab line indicates sign of motility.

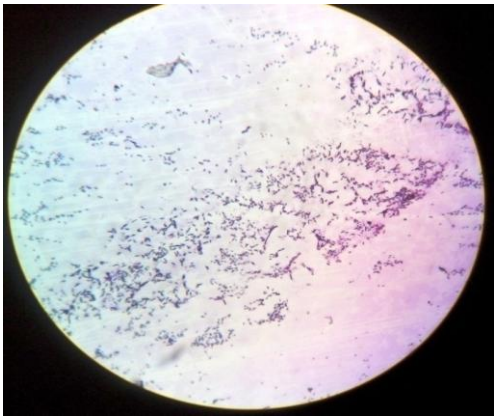


Figure 4 Microscopic photograph of gram negative and Gram positive rod shape and spiral shaped bacteria

3.2 Discussion

The obtained results suggested that the bacteria found in human oral flora are motile, catalase positive and gram negative, rods and Gram positive rods and other spiral shaped bacteria.

The ethanolic extracts of stick of *O.europaea* are inhibitory to the growth of bacteria isolated from the human oral flora. The reason for the higher inhibitory activity of Ethanolic extracts or *O. europaea* is due to the presence of bioactive metabolites which enables to inhibit the bacteria found in the human oral flora. Preparation of ethanolic extracts of *O.europaea* using disc diffusion technique is easy and cost effective.

4. Conclusion

In the present study it can be concluded that phyto-medicines are effective in treating most of the infectious bacteria's found in human oral flora and use of chewing Stick is gradually disappearing in most of the developed and developing countries due to scientific advancement and non availability of chewing sticks in Ethiopia is an outstanding exception to it. Most of the plant 2^0 metabolites serve as a plant defense mechanism against bacteria's in the oral flora.

Antibacterial activity of tested chewing Stick (*O.europaea*) extracts can be attributed to bacterial pathogens of human oral flora. The tubular reports indicated that ethanolic extracts of sticks *O.europaea* were effective that exhibited better antibacterial activity. Hence, the detailed phytochemical investigations, antibacterial screening of secondary metabolites from this plant can yield promising antibacterial agents especially for bacteria in the human oral flora. The finding benefits not only Ethiopians but also all those who suffer from oral bacterial infections across the world.

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