*IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)*
*e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 9, Issue 10 Ver. II (Oct. 2016), PP 64-69*
*www.iosrjournals.org*

# Terminaliachebula– A Potential Natural Herbal Drug Against Mastitis Isolates.

Dinesh M.D[1],Ashitha Carmel .P.P [2],Neethu George[1], Ajma. N [3], Anjana.J.C[4], S. Meenatchisundaram[5]

[1] *Department of Microbiology,Pazhassiraja College, Pulpally, Wayanad, Kerala, South India*
[2]*Department of Microbiology,Mar Athanasious College, Kothamangalam, Ernakulam.*
[3]*Department of Microbiology, E.M.E.A. College of Arts and Science,Kondotty,Malappuram, Kerala, South India.*
[4]*Department of Microbiology,SNGIST Arts and Science College, Mannakapady, Kuramallor Cochin, Kerala.*
[5]*Department of Microbiology, Nehru Arts and Science College,Thirumalayampalayam, Coimbatore – 641 105, Tamil Nadu, India.*

***Abstract:*** *Phyto-synthesis of medicines in the era of google is gaining huge importance because of its low toxicity, cost effective and eco friendly nature. In the present study,Terminaliachebula extract against the pathogens (Escherichia coli, Klebsiellaspp, and Staphylococcusaureus) isolated from the milk samples of mastitis caused cows. The main aim is to develop a potential natural herbal medicine against mastitis isolates from an environmentally friendly manner. For fulfilling this aim, the plant extract was screened by TLC examination followed by column chromatography for the isolation of inhibitory compounds of T.chebulaextract against the pathogens. Gas chromatography-Mass spectrometry (GCMS) analysis revealed the presence of Dichloroacetic acid, undec-2 enyl ester , Bis(2-ethylhexyl) phthalate and 13-Tetradecenaland 2-Ethylacridine. The Staphylococcus spisolatescomes from unhygienic practices and via milkers hand. E. coli and Klebsiella origin takes place from contaminated environment and poor hygienic condition and it causes infection in udder via gaining entry through teat canal. Phytomedicines derived from T. chebula have shown great promise in the treatment of mastitis.*

***Keywords:****GCMS Analysis, Mastitis,Minimum Inhibitory concentration, Minimum Bactericidal Concentration, and Terminaliachebula.*

## I. Introduction

Mastitis is a multi-etiological complex disease which is characterized by physical, chemical and bacteriological changes in milk and pathological changes in glandular tissue of udder (1). Mastitis is a multifactorial infection commonly affected in cattle's. However, most infections are caused by various species of gram positive and gram-negative rods, especially lactose-fermenting organisms of enteric origin, commonly termed coliforms. From an epidemiologic standpoint, the primary sources of infection for most pathogens may be regarded as contagious.

Drug allocation after intra mammary administration may not be sufficient because of widespread fibrosis and micro abscess formation in the gland; it is critical to assess the cow's immune status from a viewpoint of duration of infection, number of quarters infected, and other variables. The use of antimicrobials for a long period of time has triggered the development of multidrug resistance strains of several bacterial species. This results in the use of higher dose of antimicrobials, causing the danger of increasing amounts of drug residues in milk that causes a potential biohazard. (2) Thus, the use of medicinal plants may present a cheaper and sustainable alternative to synthetic medicines.

Medicinal plant lives are rich in secondary -metabolites and are furtive resource of drugs. These medicinal plants have large group of economically essential plants which offer basic raw materials for medicines, perfumes, flavours and cosmetics. Medicinal plants hold rich source of antimicrobial agents and broadly used as conventional medicines. Most of the peoples used crude plant extracts as herbal medicine for the treatment of most of the bacterial infection diseases. Moreover the action and efficacy of medicinal plants is still needed to be validated scientifically.

*Terminaliachebula*is a moderate tree with valuable medicinal applications used as traditional medicines. It belongs to the family Combretaceae and is commonly called as Black myrobalan, Ink tree (or) *Chebulicmyrobalan*. It is extensively used in unani, ayurveda and homeopathic medicine. *Terminaliachebula*is a popular traditional medicine not only used in India but also in other countries of Asia and Africa.It is used as an antioxidant, neuroprotective drug and for treatment of heart disease, inflammation, brain dysfunction. It is used as an anti-aging agent and it is found to improve the mental abilities. The plant also has adrenergic function and

helps to recover from stress. With sugar water it is used to treat opthalmia, skin itching and oedema. SumitVarshney(2012) reported the antibacterial effect of *Terminaliachebula* against mastitis field isolates. In our present research work we investigated the *in vitro*inhibitory activity of *Terminaliachebula* against mastitis causing pathogens.

## II. Materialsand Methods

### 2.1. Isolation and Identification of Mastitis causing organisms milk samples

Raw milk samples were collected in sterile sample bottles from various veterinary dispensaries in and around Pulpally, Wayanad, Kerala. The bacterial species for the study were isolated from the collected milk samples. The bacterial isolates were identified by cultural and physiological, morphological and biochemical tests according to Bergey's manual of Determinative Bacteriology (3).The Hanging drop method was employed for the detection of the motility of the organisms.

### 2.2. Preparation of Plant Extracts

The medicinal plant obtained from in and around Wayanad region wasused for the study. The plant extract was prepared by the method of (4) using distilled water as the solvent. About 20 g of powdered sample of the herb was extracted by soaking in 180 mL of distilled water in a beaker, stirred for about 6 min and left overnight. Thereafter, the solution was filtered using filter paper (Whatman No. 1) and the extracts were evaporated to dryness under reduced pressure below 40°C.The crude plant extract was subjected to phytochemical analysis for detecting the chemical compounds in it(5, 6).

### 2.3. Antimicrobial activity of plant extracts against clinical mastitis pathogens

#### 2.3.1. Well diffusion method

The agar well diffusion method was adopted according to Kavanagh, (7) to assess the antibacterial activity of the prepared extract. A loop full of bacterial stock suspensions was thoroughly mixed with 100 ml of sterile nutrient agar and kept for overnight incubation. 0.1ml of overnight culture was spread on the surface of Muller Hinton agar plates and the wells were cut.The wells were filled with extract of various concentrations of about 20µl to 80 µl. After 24 hours at $37^0$C, the agar plates were examined for the zone of inhibition and the zones were measured in millimeters.The zones were measured, averaged and the mean values were tabulated.

#### 2.3.2. Minimum Inhibitory Concentration (MIC) – Dilution Method

Different concentrations of plant extract ranging from 1mg to10mg/ml were added into test tubes which containing 1ml nutrient agar. To this 50µl of an overnight broth culture of the test organisms were inoculated and incubated the tubes for 24 hours at $37^o$C. A tube containing 5 ml sterile nutrient broth was inoculated with the drop of an overnight broth culture and kept at $4^o$C in a refrigerator overnight, to be used as standard for the determination of complete inhibition.

#### 2.3.3. Minimum Bactericidal Concentrations (MBC)

Dilutions and inoculations were prepared in the same manner as described for the determination of MIC. The control tube without plant extract is immediately sub cultured (Before incubation) along with the tubes with plant extract at $37^o$C overnight. The MIC of the control organism was read to check that the drug concentrations are correct. The growth was compared with control tube, which represents the original inoculum. If a similar number of colonies presents it indicates bacteriostatic only. A reduced number of colonies-indicates a partial or slow bactericidal activity and if no growth was observed, thenthe whole inoculum has been killed. The highest dilution showing at least 99% inhibition is taken as Minimum Bactericidal Concentrations (MBC).

### 2.4. Thin layer Chromatography

Prepared silica (Silica gel G, Hi Media of 60 to 120 meshes)was spotted with 10 µL extracts using capillary tubes. The solvent system used as mobile phase was Ethanol, Methanol, Ethyl acetic acid (50:50:1µl). Following Thin Layer chromatography bands appeared and was visualized in the UV - chamber at a wavelength of 254 NM. The spot is scraped using sterile spatula and dissolved in solvent systems. After centrifugation the supernatant which containing the plant compounds was collected and performed well diffusion method for assessing inhibition properties of separated compounds. **(**8, 9)

### 2.5. **Column Chromatography**

After assessing the suitable solvent system for getting maximum separation via TLC examination, [Ethanol, Methanol, Ethyl acetic acid (50:50:1µl)] was used to grain the silica packs tightly. The *T. Chebula* extracts dissolved in solvent (same as in the column) was added to the column using a Pasteur pipette. The

solvent was continually added to the top of the column until each band resolves and was carefully collected. The collected fractions were examined for inhibition properties of eluted fractions.

**2.6. GC-MS analysis**

Based on the inhibitory properties of fractions collected after column chromatography, fraction 1 and 8 were subjected for GC MS analysis. Chromatographic separation was carried out by Agilent Technologies-7890 GC System, 5975C inert MSD. The column used was Agilent 190913-433; 325$^{o}$C capillary column measuring 30mX250µm with a film thickness of 0.25µm. The carrier gas used was Helium at a flow rate of 1.1 ml/min. 1µl sample injection volume was utilized. The inlet temperature was maintained at 100 - 250°C. The mass spectra of compounds in samples were obtained by electron ionization (EI) at 70 eV, and the detector operated in scan mode from 20 to 600 amu. Identifications were based on mass spectral matching with standard compounds in NIST and Wiley libraries. The essential chemical constituents were identified by matching mass spectra with spectra of reference compounds in mass spectral library of National Institute of Standards and Technology (NIST 147). The relative amounts of individual components were expressed as percent peak areas relative to total peak area.

## III. Result

**3.1. Table1:** Morphological characterization of Collected Milk samples

| | Samples | Morphology characterisation |
|---|---|---|
| **1** | Sample 1 | Gram negative rods |
| **2** | Sample 2 | Gram negative rods |
| **3** | Sample 3 | Gram negative rods |
| **4** | Sample 4 | Gram negative rods |
| **5** | Sample 5 | ------ |
| **6** | Sample 6 | Gram positive cocci |
| **7** | Sample 7 | Gram negative rods |
| **8** | Sample 8 | Gram negative rods |
| **9** | Sample 9 | -- |
| **10** | Sample 10 | --- |

**3.2. Table 2:** Biochemical characterization

| Sample | Indole Test | Methyl red | VogesProskauer Test | Citrate utilization Test | Urease Test |
|---|---|---|---|---|---|
| Sample 1 | + | + | - | - | - |
| Sample 2 | - | - | - | + | - |
| Sample 3 | + | + | - | - | - |
| Sample 4 | + | + | - | - | - |
| Sample 5 | | | | | |
| Sample 6 | | | | | |
| Sample 7 | - | - | - | + | - |
| Sample 8 | + | + | - | - | - |
| Sample 9 | | | | | |
| Sample 10 | | | | | |

**3.3. Table: 3** Phytochemical result

| Tests | *TerminaliaChebula* |
|---|---|
| **Saponin** | + |
| **proteins** | - |
| **Tannins** | + |
| **Anthraquinone** | + |
| **Flavanoids** | - |
| **Phenols** | + |
| **Salkowsky test** | + |
| **Anthrone test** | - |

**3.4. Table: 4 -** Antibacterial activity of crude extracts of various medicinal plants against E. coli, *Klebsiella* and *Staphylococcus aureus*-Well cut Method

| Plant extracts | Conc. µl/ml | Zone of inhibition (mm) | | |
|---|---|---|---|---|
| | | *E.coli* | *Klebsiella* | *Staphylococcus aureus* |
| *TerminaliaChebula* | **80** | **11.5** | **10.4** | **11.2** |
| | **60** | **9.8** | **8.2** | **10.4** |
| | **40** | **6.9** | **5.3** | **7.4** |
| | **20** | **5.2** | **4.2** | **6.2** |

**3.5. Table 5:** Minimal inhibitory concentrations (MIC) and Minimum Bactericidal Concentrations (MBC)

| **Plant extracts** | **MIC/MBC** | **Aqueous Extract - *MIC (mg/ml)*** | | |
|---|---|---|---|---|
| | | ***E.coli*** | ***Klebsiella*** | ***Staphylococcus aureus*** |
| *Terminaliachebula* | MIC | 2 | 3 | 2 |
| | MBC | 5 | 6 | 4 |

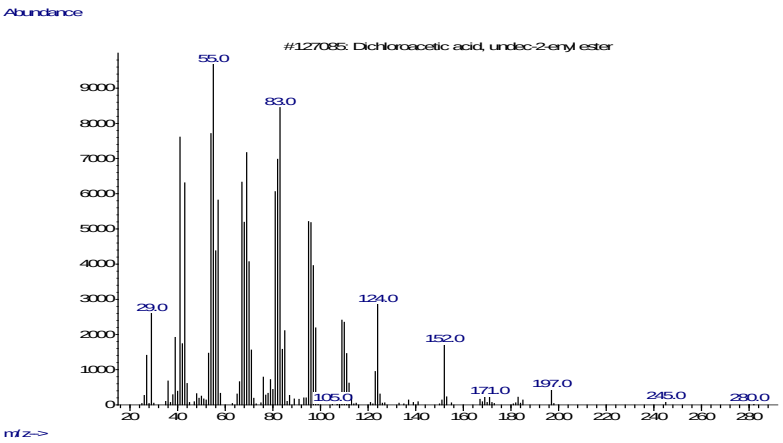


**Figure1:** Total ion chromatograme of Fraction – 1

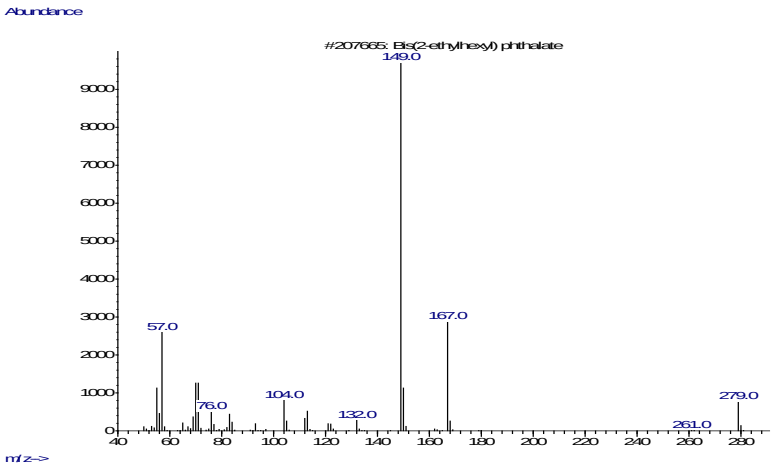


**Figure 2:** Total ion chromatograme of Fraction – 1

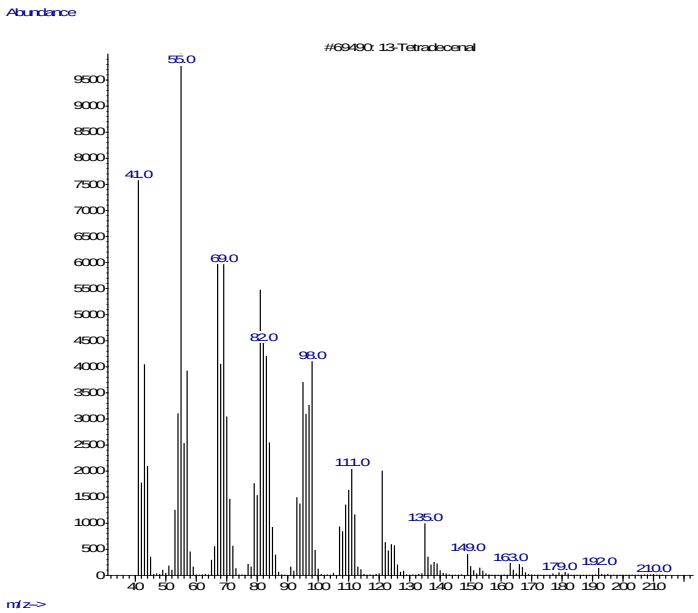


**Figure3:** Total ion chromatograme of Fraction – 8

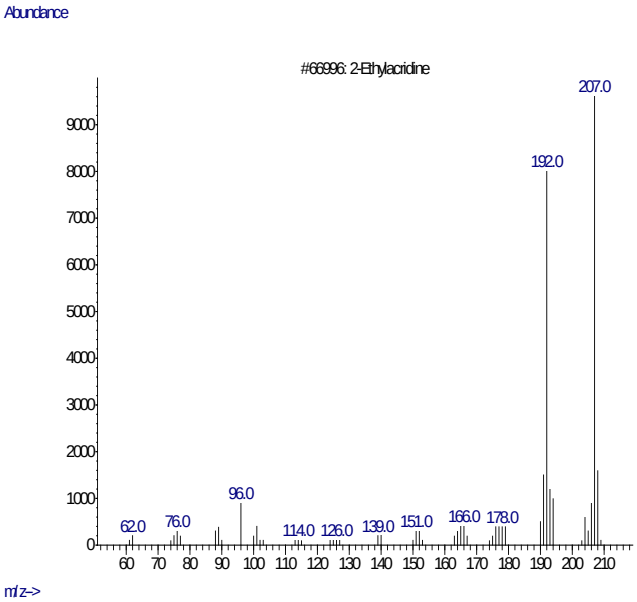


**Figure4:** Total ion chromatogrameof Fraction – 8

The medicinal plants obtained from in and around Wayanad region were used for the present study. The plant was selected based on the oral literature and from the suggestions of dairy farmers in WayanadDistrict. The preliminary screening and identification of bioactive chemical element in the *T. Chebula* were carried out. In the current study, the presence of phytochemicals, Saponin, Tanin, Antraquinone and Phenols were detected (Table- 3). Mastitis causing coliform pathogens was isolated from milk samples from the clinical cases of mastitis in cow, in and around wayanad region. Following the collection of the milk samples, the isolation and identification of bacteria were made on the basis of morphological(Table –1), biochemical characterization (Table – 2) and cultural methods. Based on the results obtained from these tests the isolates were identified as *Escherichia coli, Klebsiella species*and*Staphylococcusaureus*.

According to the result obtained from collection, isolation and identification the seasonal mastitis illness rate is extremely affected by *Escherichia coli, Klebsiella*in the winter season in the period of November 2015 – February 2016. The most prominent activity with inhibition zones of more than 10 mm was shown by *TerminaliaChebula* (inhibition zone 11.5 mm against all pathogens at a concentration of *80*µl/ml). When the concentration of the extracts was decreased slight decrease in inhibition zones were observed (Table – 4).Based on the TLC results *Terminaliachebula* extracts were subjected for column chromatography using silica gel as adsorbent. The sample was eluted using the same solvent later used in TLC 8 fractions were collected at the flow rate of 0.6ml/minute and fractions were concentrated at room temperature. The fraction 1 and 8 showed the inhibition properties against mastitis isolates. These fractions were further subjected to GCMS analysis. The quantitative determination of the chemical compounds was based on the comparison of peak areas of samples with those in GC MS library. (Figure 1-4).

## IV. Discussion

In the present work, investigation was performed inorder to detect the main pathogens affecting udder health and to find an antibacterial phyto-constituent against it. About3 different clinical pathogens in various milk samples were isolated and the isolates were identified as *Escherichia coli, Klebsiellaspp, and Staphylococcus aureus*after the morphological, biochemical and cultural characterization. The present finding are in accordance with the finding of (10) where they tested 60 milk samples out of which 40% *Staphylococcus*, 16 % *Streptococcus*, 20% *Escherichia coli*, and 10% *Klebsiella* organisms were isolated from milk samples. Mastitis caused by staphylococci is most prevalent and important in context to India. *Staphylococcus*sppcomes from the unhygienic practices and via milkers hand. *E. coli* and *Klebsiella* origin takes place from contaminated environment and poor hygienic condition and it causes infection in udder via gaining entry through teat canal (11).Phytomedicines derived from plants have shown great promise in the treatment of intractable infectious diseases. A special feature of higher plants is their capacity to produce a large number of secondary metabolites. The plant specimen *Terminaliachebula* was collected from the Wayanad district. Each plant extracts were tested at three different concentrations (20,40,60 and 80µl/ml) to see their inhibitory effects against mastitis isolated pathogens. The most prominent activity with inhibition zones of more than 11.5 mm was shown by *Terminaliachebula* followed by 7.8mm. From earlier investigation an interesting results was found that *Terminaliachebula* inhibited the growth of both strains *E. coli* and *S. aereus* with highest zone of

inhibition12mm and 13mm respectively with 15% methanolic and aqueous extracts. *Terminaliachebula* was also found active with all concentrations for aqueous and methanolicpreparations (12). The TLC analysis revealed the presence of active constituents. The plant extract in column chromatography revealed the presence of eight fractions out of which fraction 1 and 8 showed antibacterial activity against mastitis causing clinical samples. The fractions were further investigated under GCMS analysis. On this examination, fraction 1 revealed 2 major components Dichloroacetic acid, undec-2 enylesterandBis(2-ethylhexyl) phthalate. In fraction 8, the compounds detected were 13-Tetradecenaland 2-Ethylacridine. In an earlier investigation it was reported that an ester of phthalate used as plasticizers in detergent as base and in aerosol spray (13), di-(2-ethylhexyl) phthalate, a major bioactive metabolite proved with antimicrobial and cytotoxic property (14).13 tetradecenal is a non - methyl ester having inflammatory along with 2- ethyl acridine.

The mastitis milk is of poor bacteriological quality and hazardous for human consumption. Also the mastitis infection should be controlled in animals using natural products compared to antibiotics since their residual amount would be present in milk for a long time which would be consumed by humans. To control the mastitis infections, hygienic practices of farm and environment should be implemented. These findings highlight the need to devise improved hygiene practices and to apply actual monitoring throughout the production to delivery chain along with proper control measures.

## References


[1] Radostitis, O.M., Gay, C.C., Blood, D.C. and Funchcliff, KW. (2000) Veterinary medicine: *Atext book of the disease of cattle, sheep, goat, horse and pigs. 9th Edition*W.B.Saunders, London.

[2] AnnapooraniChockalingam, Dante S. Zarlenga, Douglas D. Bannerman., Antimicrobial activity of bovine bactericidal permeability-increasing protein-derived peptides against gram-negative bacteria isolated from the milk of cows with clinical mastitis. American Journal of Veterinary Research.,68 (11): 2007, pp: 1151 – 1159.

[3] Holt, J. G., Bergey, D. H., Krieg, N .R. (1984). *Bergey's Manual of Systematic Bacteriology, Vol2,* Williams and Wilkins,Baltimore, USA. pp 408-423.

[4] Uhegbu, F.O., I. Elekwa and C. Ukoha, 2005. Comparative Efficacy of crude Aqueous Extract ofMangiferaindica, carica papaya and sulphadoxinepyrimethamine on the mice infested with malariaparasitein vivo. *Global J. Pure Appl. Sci., 11*: 399-401.

[5] Trease, G.E., Evans, W.C., 2002. *Pharmacognosy, 15th ed*. Springer, Berlin.

[6] Sofowora, A. 1993. Screening plants for bioactive agents. In: *Medicinal Plants and Traditional Medicinal in Africa, second ed.*, Spectrum Books Ltd., Sunshine House, Ibadan, Nigeria, pp. 134– 156.

[7] Kavanagh, F. (1972). *Analytical Microbiology, vol. 2,* Academic Press, New York.

[8] Eagon Stahl. (1963). Thin Layer Chromatography.A laboratory handbook.Academic press, New York.

[9] Chatwal R. Gurdeep. Sham K. Anand. *Instrumental Methods of Chemical analysis*, Himalaya Publishing House reprint, 2007; 2: 612-2.614.

[10] Sumathi B.R., Veeregowda B.M. and Amitha R., Gomes. 2008. Prevalence and antibiogram profile of bacterial isolates from clinical bovine mastitis. *Veterinary World 1(8):* 237-238.

[11] Mallikarjunaswamy M.C. and Murthy G.V.K. 1997. Antibiogram of bacterial pathogens isolated from bovine subclinical mastitis cases. *Indian Veterinary Journal 74:* 885-886.

[12] Sachin Kumar, Hotam Singh Choudhary, ChandrabhanSeniya, 2011. In vitro antibacterial study of aqueous and methanolic extracts of some selected medicinal plants. *J. Chem. Pharm. Res., 2011, 3(4):*854-860.

[13] Ghorpade N, Mehta V, Khare M, Sinkar P, Krishnan S, Rao CV. Toxicity study of diethyl phthalate on fresh water fish Cirrhinamrigala. Ecotoxicol Environment Saf. 2002; 53 (2): 255-258.

[14] Sayed MH. Di-(2-ethylhexyl) Phthalate, a major bioactive metabolite with antimicrobial and cytotoxic activity isolated from the culture filtrate of newly isolated soil Streptomyces (Streptomyces mirabilis Strain NSQu-25) *World App Sci J. 2012; 20 (9):* 1202-1212.