EVALUATION OF ANTIMICROBIAL EFFECTS OF TEA EXTRACTS ON BOVINE MASTITOGENIC ISOLATES

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BY
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CERTIFICATE – I

This is to certify that the thesis entitled ***" Evaluation of antimicrobial effects of tea extracts on bovine mastitogenic isolates "*** *submitted in partial fulfillment of the requirement for the degree of Master of Veterinary Science in Veterinary Microbiology of the Orissa University of Agriculture and Technology, Bhubaneswar is a faithful record of bonafide research work carried out by* ***Susmita sethi*** *under my guidance and supervision.*

This is further certified that no part of the thesis has been submitted for any other degree or diploma.

This assistance and help received from different sources during the course of study have been fully acknowledged.

(Dr. Rajashree Mishra)

Advisor

CERTIFICATE - II

This is to certify that the thesis entitled ***" Evaluation of antimicrobial effects of tea extract on bovine mastitogenic isolates " submitted by Susmita Sethi to the Orissa university of agriculture and technology, Bhubaneswar*** *in partial fulfillment of the degree of* ***Master of Veterinary Science*** *in the subject of* ***Veterinary Microbiology*** *has been approved by the student 's. advisor committee after an oral examination on the same in collaboration with an external examiner.*

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(Susmita Sethi)

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CONTENTS

List of abbreviations

Ast	- Antibiotic sensitivity test
@	-At the rate
%	-Percent
BA	- Blood Agar
BHIB	-Brain Heart Infusion Agar
CFU	- Colony Forming Unit
Cm	- Centimeter
Conc	- Concentration
C°	- Degree Centigrade
Dia	- Diameter
EVM	-ethno veterinary medicine
Fig	-Figure
Gm	-Gram
H_2S	-Hydrogen Sulphide
Hr	- Hour
i.e	-That is
Lit	- Litre
Mcg	- Microgram
MI	-Millimeter
MBC	-minimum bactericidal concentration
MIC	-minimum inhibitory concentration
Min	-Minite
MLA	- Mc Conkeys lactose Agar
Mm	- Milimeter

µm	- Micrometer
MR	-Methyl Red
N	-Normality
NA	-Nutrient Agar
NB	- Nutrient Broth
NaCl	-Sodium chloride
NaOH	-Sodium hydroxide
No	- Number
NO_2	- Nitrite
NSS	- Normal Saline Solution
OB	- Out Break
Pic	- Picture
Ph	- Hydrogen ion concentration
US	- United states
VP	- Vogers proskaeur
+Ve	- Positive
-Ve	- Negative
Viz	- Namely

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I. INTRODUCTION

Bovine mastitis is an inflammatory condition of mammary gland most often caused by bacterial intramammary infection, resulting in significant economic losses to the dairy industry. Among cattle diseases, bovine mastitis is a serious problem which affects the basic income of the farmers depleting their dairy sources. Worldwide, mastitis is associated with economic losses of $35 billion annually. It adversely affects milk production whereby losses due to subclinical mastitis are more severe than those due to clinical cases. Controlling subclinical mastitis can reduce the losses in milk production substantially. Decreased milk production and quality, as well as veterinary expenses, all contributes to these economic losses (Chockalingam *et al.,* 2007). The increased production costs associated with mastitis can be attributed to culling, medication, discarded milk, and reduced milk quality.

Mastitis is also associated with number of zoonotic diseases in which milk acts as a vehicle of infection. Recently, about 20,582 mini dairy farms each with five or more high yielding cross-bred cows have been established in private sector in Bangladesh and most of these dairy farms are confronted with problems of clinical and subclinical mastitis. The clinical mastitis can be diagnosed on history and clinical findings but laboratory examinations are required to ascertain the subclinical mastitis. The prevalence of subclinical mastitis has been shown to be 15 to 40 times more than the clinical mastitis. The prevalence of bovine mastitis is higher in farms with larger herd sizes than in those with lower herd sizes (Radostits *et al.,* 2000). The prevalence is also higher in cows with lesions and tick infestations on the skin, udders and teats than cows without these factors, in early lactation stage than in the mid-lactation stage .

Epidemiological studies on mastitis revealed that mastitogenic organisms are widespread on different body sites of the cows, milker's hands, milking cans and in the milk samples. Teat apices have been reported to be the most common site, from where these organisms have been isolated. The mastitis causing organism, *Staphylococci*, the chief udder pathogen, has been isolated from almost all the body site examined and environment but *Streptococci* from fewer body sites, whereas the prevalence of *Escherichia coli* has been reported to be widespread. The prevalence of subclinical mastitis in milch cow have been reported to be 16.5% with Whiteside Test (WST) and 15.8% with California Mastitis Test (CMT) from Baghabarighat, Sirajgonj district by. *Staphylococci* are the major etiological agents, followed by *Streptococci* and *Escherichia coli* causing subclinical mastitis in cows in India but only *Staphylococci* have been isolated from subclinical mastitis cases of cows from Bangladesh.

Clinical and subclinical cases of mastitis are routinely treated with antimicrobials both intramammarily and parenterally. The use of antimicrobials over long periods has triggered the development of multidrug resistant strains, which has resulted in the use of increasing doses of antimicrobials, causing the danger of increasing amounts of drug residues in milk, a potential biohazard (Dhanabalan *et al.*, 2008).An infected quarter is the source of contagious pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*, whereas environmental pathogens such as *Escherichia coli, Streptococcus dysgalactiae*, and *Streptococcus uberis* originate from a variety of sources including bedding, manure, pastures, and pond water. Bacteria gain access to a healthy gland most frequently during and after the milking process, when vacuum fluctuations, liner slips, and relaxed teat canal sphincter muscle tone afford the greatest opportunity for invasion.

Staphylococci are the most important and prevalent mastitis causing organism globally, including India. Higher incidence of *Escherichia coli* mastitis may be due to poor hygienic conditions, as *Escherichia coli* originates from the cow's environment and infect the udder via the teat canal (Mallikarjunaswamy and Murthy ,1997). Environmental bovine mastitis caused by *Coliform* bacteria has increased in many herds and countries. *Escherichia coli* constitute the majority of these *Coliform* bacteria. *Escherichia coli* originate from the cow's environment and infect the udder via the teat canal (Eberhart, 1979). Many authors have reported *Escherichia coli* as the second most common etiological agent causing mastitis in cows following *Staphylococcus aureus*.

Higher incidence of *Escherichia coli* mastitis may be due to poor hygienic conditions or intensive use of antimicrobials targeted against Gram positives for mastitis control (Radostits *et al.*, 2000). As a result *Coliform* mastitis remains as one of the most difficult diseases to treat in the modern dairy industry. Curative therapy with antibiotics remains only moderately effective and depends on the stage at which the disease is treated. The most important factor in the control of *Escherichia coli* mastitis is the emergence of multiple drug resistant strains. Indiscriminate use of antibiotics in the treatment of mastitis has lead to the emergence of drug resistant strains. Furthermore, the transmissibility of antimicrobial resistance and virulence factors by conjugation may contribute to the development and dissemination of pathogenic *Escherichia coli* strains (Holmberg *et al.*, 1984).

Mastitis in bovines has become extremely complex and the costliest disease in India. It affects 50% of the herd population. It has been estimated that the mastitis alone can cause approximately 70% of all avoidable losses incurred during milk production. One important reason for treatment failure is assumed to indiscriminate use of antibacterials without testing in vitro sensitivity of causal

organisms. This practice at one hand increases economic losses and on other results in development of resistance to commonly used antimicrobials.

Intramammary infusion of antibiotics is the most common treatment method available for treating mastitis. However, the cure rates obtained with antibiotics are generally poor and vary for different mastitis pathogens. For example, the cure rates of mastitis caused by *Staphylococcus aureus* range from 20 to 75%. Use of antibiotics against bacterial diseases in cattle, including mastitis, may potentially lead to the emergence of antibiotic resistant strains of bacteria (Berghash *et al.*, 1983; White, 1999). Moreover, the use of antibiotics to treat bovine mastitis has been implicated as a common source of drug residues in milk. Approximately 90% of the residues detected in milk over a period of 5 yr in Michigan originated from antibacterial therapy for mastitis (Erskine *et al.*, 2003).

For over a decade, the pace of development of new antimicrobial agents has slowed down while the prevalence of resistance has grown at an astronomical rate. The rate of emergence of antibiotic resistant bacterial is not matched by the rate of development of new antibiotics to combat them (Prescott and Klein, 2002). Today, multiple antibiotic resistance among bacterial pathogens is a major public health problem worldwide (Deguchi *et al.*, 1998). It is making a growing number of infections difficult to treat and infections more rampant and deadly (Tzouvelekis *et al.*, 1998).

Usually, infections resulting from strains that are resistant to main groups of antibiotics like the β-lactams and aminoglycosides are treatable with vancomycin, chloramphenicol or other antibiotics (Hugo and Russell, 2003). But resistance has been developed to these drugs over the years. In particular infections due to *Staphylococcus aureus* have continued to be a major source of morbidity and mortality and these organisms are now exhibiting multi-drug resistance to commonly used antibiotics, hence a significant cause of concern among physicians

(Luck et *al.*, 1998; Tzouvelekis *et al.*, 1998). The presence of efflux pumps and multidrug resistance (MDR) proteins in antibiotic resistant organisms contribute significantly to the intrinsic and acquired resistance in these pathogens.

The need to combat microbial resistance to antibiotics is an increasing global concern (Kunin, 1993; Twomey, 2002). *Staphylococcus aureus* is one of the gram-positive microorganisms that have been shown to exhibit resistance to a wide range of commonly available antibiotics, especially the penicillins (Ghobashy *et al.*, 1994; Haldane and Affias, 1981). Therefore, penicillins are often administered in combination with other antibiotics in the treatment of resistant (or suspected resistant) bacterial infections (Wise *et al.*, 1969; Okore, 1990). Combined antibiotic therapy has been shown to delay the emergency of bacteria resistance and may also produce desirable synergistic effects in the treatment of bacteria infection (Elopoulos, 1982). In Nigeria, antibiotics are sometimes willfully or inadvertently administered concomitantly with herbs or beverages (Nwafor *et al.*, 2003; Esimone *et al.*, 2003a, b). Combination therapy can be used to expand the antimicrobial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity, and to obtain synergistic antimicrobial activity (Pankey and Ashcraft, 2005).

Tea from the leaves of plant *Camellia sinensis* has been shown to have wide range of antioxidant, antiinflammatory, anti-carcinogenic and antibacterial activity against many pathogens. (Hamilton Miller, 1995). *Staphylococcus aureus, Vibrio parahemolyticus, Clostridium perfringens, Bacillus cereus, Pleisomonas shigelloides* and *Aeromonas sobria* failed to grow in tea normally consumed by Japanese people. Tea components also inhibit the growth of *Vibrio cholera* O1, *Streptococcus mutans*, *Shigella dysenteriae* and other bacteria grown in vitro. Susceptibility of bacterial strains to the tea extract has been shown to be related to

differences in cell wall components. Several studies have also shown that monoterpenes exert membrane-damaging effects.

In the light of the aforementioned problems and concerns, there is a need for alternative approaches for controlling mastitis in dairy cows. Plant-derived essential oils represent a group of natural antimicrobials that have been traditionally used to preserve foods as well as enhance food flavor. The antimicrobial properties of several plant-derived essential oils have been demonstrated. A number of compounds with an in vitro activity of reducing the MICs of antibiotics against resistant organisms have also been isolated from plants. Polyphenols (epicatechingallate and catechingallate) have been reported to reverse β-lactam resistance in Methicillin Resistant *Staphylococcus aureus* (MRSA). Diterpenes, triterpenes, alkyl gallates, flavones and pyridines have also been reported to have resistance modulating abilities on various antibiotics against resistant strains of *Staphylococcus aureus* (Marquez *et al.*, 2005; Oluwatuyi *et al.*, 2004).

Medicinal plants have been used for ages in developing countries as alternative treatment to health problems. India has a diverse flora and a rich tradition in the use of medicinal plants for antimicrobial applications. In India specifically in Tamil Nadu ethno-veterinary practices are very common in villages. Most of the approaches of the farmers are based on empiric knowledge with significant results in cattle. A short survey prior to this study was undertaken among known farmers about their interest in ethno-botany and treatment of their cattle sources. Most of them expressed a desire to learn more about the proper use and application of ethno-veterinary practices as these were economically, socially and culturally more acceptable for marginalized communities.

Moreover, plant-derived antimicrobials have been reported not to induce resistance in gram-positive and gram-negative bacteria after prolonged exposure (Ohno *et al.*, 2003; Domadia *et al.*, 2007). Combined antibiotic therapy has been shown to delay the emergency of bacteria resistance and may also produce desirable synergistic effects in the treatment of bacteria infection (Elopoulos, 1982). There are indications that some herbal materials can act as antibiotic resistant inhibitors (Gibbons *et al.*, 2003; Sibanda and Okoh, 2008; Yam *et al.*, 1998). Combinations of some herbal materials and different antibiotics might affect the inhibitory effect of these antibiotics. A few studies such as Braga *et al.*, (2005), Dickson et *al.*, (2006) and Gibbons *et al.*, (2003) have reported that plant extracts can enhance the in vitro activity of certain antibiotics against strains of MDR *Staphylococcus aureus* and other pathogens.

To chalk out suitable antibiotic therapy, bacterial isolation and antibiotic sensitivity studies are always essential. Keeping these points in view the present study was undertaken to identify the bacterial causes of mastitis and to select a suitable antibiotic for treatment.

Outline of the programme

- Isolation of the micro-organisms from the mastitic milk samples of cross bred cows from different farms and institutes.
- Identification of the organisms by
  -Morphological and cultural characterization.
  -Biochemical characterization.
- Antibiogram study of field isolates.
- Evaluation of antimicrobial effects of tea (*Camellia sinensis*) extracts on bovine mastitogenic isolates.

- Calculation of minimum inhibitory concentration (MIC) of tea (*Camellia sinensis*) extracts on bovine mastitogenic isolates.
- Calculation of minimum bactericidal concentration (MBC) of tea (*Camellia sinensis*) extracts on bovine mastitogenic isolates.
- Evaluation of synergistic effects of different antibiotics and different types of tea (*Camellia sinensis*) extracts on bovine mastitogenic isolates.

II. REVIEW OF LITERATURE

MASTITIS – THE DISEASE AND ECONOMIC ASPECTS:

Murphy and Stuart (1953); Treece *et al.*(1966) and Frost (1967); studied on the fibrous proteins of keratin in the teat canal which bind electrostatically to mastitis pathogens, which alter the bacterial cell wall, rendering it more susceptible to osmotic pressure. Inability to maintain osmotic pressure causes lysis and death of invading pathogens.

Scham *et al.* (1971) observed that the infection rate in cows with teat lesions is more than cows with normal teats. Cows with disk-shaped, inverted, pointed and round shaped teat ends have 88.46 %, 61.54 %, 54.17 % and 40.86 % rates of infection, respectively. The pendulous udder exposes the teat and udder to injury and pathogens may easily adhere to the teat and get access to the gland tissue. The internal environment of the mammary gland which is favourable for the multiplication of invading bacteria. The by-products of bacterial growth and metabolism irritate the delicate tissues of the glands causing inflammation.

Schultz *et al.* (1978) studied on various forms of clinical and sub clinical mastitis that occur in bovines. In the clinical mastitis all the five cardinal signs of udder inflammation (redness, heat, swelling, pain and loss of milk production) are present, while the sub-clinical form is bereft of any obvious manifestation of inflammation. Sub-clinical mastitis is 3-40 times more common than the clinical mastitis and causes the greatest overall losses in most dairy herds.

Chaudhry and Khan (1978) studied on the statistics of losses due to mastitis in Punjab alone, the total losses caused by clinical mastitis amount to Rs. 240 million per year. These occur through discarded milk, reduction in milk yield, premature culling of animals and replacements. The losses caused by clinical mastitis do not take into account those caused by sub-clinical mastitis which is less obvious and may only be detectable by measuring the milk's somatic cell counts (SCC).

Eberhart (1979); Sing and Buxi (1982); Anwar and Chaudhary (1983) pointed out that environmental bovine mastitis caused by *Coliform* bacteria has increased in many herds and countries. *Escherichia coli* constitute the majority of these *Coliform* bacteria. *Escherichia coli* originate from the cow's environment and infect the udder via the teat canal. *Staphylococci*, *Streptococci*, *Escherichia coli* and *Pseudomonas* are found in buffaloes suffering with mastitis. Resistance to penicillin among staphylococci isolated from mammary glands is wide spread.

Weber *et al.* (1983); Bramley and Dodd (1984) analysed that because of indiscriminate ingestion of fat, casein and milk components, the mammary gland macrophages are less effective at phagocytosis than are blood leukocytes. The teat end prevents milk from escaping, and bacteria from entering into the teat. From inside, the teat canal is lined with keratin derived from stratified squamous epithelium. Damage to keratin has been reported to cause increased susceptibility of teat canal to bacterial invasion and colonization.

Cady *et al.*(1983) and Khan *et al.* (1991) reported that in Nili-Ravi buffaloes, mastitis shortens the lactation period of each animal by 57 days on an average and reduces 438 kg of milk per lactation. Mastitis continues to be the most costly disease of dairy animals. Field surveys of major livestock diseases in Pakistan have ranked mastitis as number one disease of dairy animals.

Holmberg *et al.*(1984) reported that the transmissibility of antimicrobial resistance and virulence factors by conjugation may contribute to the development and dissemination of pathogenic *Escherichia coli* strains. Therefore, it is necessary to select suitable antibiotics, preferably after antibiotic sensitivity testing and using such antibiotics at an adequate dose for sufficient duration to ensure effective treatment and control of *Escherichia coli* mastitis.

Gudding *et al.* (1984) and Nickerson (1989) found that mastitis is one of the major causes of economic loss in dairy cattle. According to them *Staphylococcus aureus*

is the main pathogenic species causing the subclinical form of mastitis which impairs alveolar function, reduces milk yield and has a deleterious effect on milk composition, including increased milk somatic cell count (SCC).

Sordillo *et al.* (1987) studied on bacterial pathogens which are able to traverse the opening of teat end by escaping antibacterial activities establish the disease process in the mammary gland which is the second line of defense of the host. In dairy animals, the mammary gland has a simple system consisting of teats and udder, where the bacteria multiply and produce toxins, enzymes and cell-wall components which stimulate the production of inflammatory mediators attracting phagocytes. The severity of inflammatory response, however, is dependent upon both the host and pathogen factors. The pathogen factors include the species, virulence, strain and the size of inoculum of bacteria, whereas the host factors include parity, the stage of lactation, age and immune status of the animal, as well as the somatic cell count. Neutrophils are the predominant cells found in the mammary tissue and mammary secretions during early stage of mastitis and constitute >90% of the total leukocytes.

Ratafia (1987); Mahmoud (1988); Kirk and Bartlett (1988); Ramachandrainh *et al.* (1990) said that globally mastitis leads to losses of 53 billion dollars annually. Schalm test was more reliable than other test for detection of mastitis. Subclinical mastitis which causes reduction in milk quality, quantity and market value of milk and thereby responsible for up to 70 % of the losses in mastitis. Clinical mastitis and found that it causes enormous losses for breeders and consequently influences the national income of the country.

Murphy *et al.*(1988) stated that mastitis in dairy animals occurs when the udder becomes inflammed and bacteria invade the teat canal and mammary glands. These bacteria multiply and produce toxins that cause injury to the milk secreting tissue, besides, physical trauma and chemical irritants. These cause increase in the

number of leukocytes, or somatic cells in the milk, reducing its quantity and adversely affecting the quality of milk and milk byproducts. The teat end serves as the first line of defense against infection. From outside, a sphincter of smooth muscles surrounds the teat canal which functions to keep the teat canal closed.

Sordillo and Nickerson (1988) studied that macrophages are the predominant cells found in milk and tissue of healthy involuted and lactating mammary glands Macrophages ingest bacteria, cellular debris and accumulated milk components. The phagocytic activity of macrophages can be increased in the presence of opsonic antibody for specific pathogens.

Watts (1988) identified 137 microbial species as causative agents of bovine mastitis, including agents involved in its pathogenesis. However, viruses were not included, mastitis research has concentrated on bacterial pathogens because in case of viral infections, signs of mastitis may not have been recognised because other clinical signs were more prominent. Subclinical mastitis cases are often not diagnosed and consequently their aetiology is not investigated. This may cause an underestimation of virus infections involved in bovine subclinical mastitis.

Owens and Watts (1988) studied the drug resistance mediated by penicillinase production. These organisms have been shown to act as donors of antibiotic resistance genes for *Staphylococcus aureus*. A detailed knowledge of the resistance determinants and biochemical characteristics of staphylococci is important not only in assessing the clinical significance of these organisms, but also in their epidemiological typing.

Radostits *et al.* (1994) found that bovine mastitis is generally considered to be of infectious nature leading to inflammation of one or more quarters of the mammary gland and it is often affecting not only the individual animal but the whole herd or at least several animals within the herd. If left untreated, the condition can lead to deterioration of animal welfare resulting in culling of affected cows, or even death.

Mastitis-causing pathogens include bacteria and non-bacterial pathogens, like mycoplasms, fungi, yeasts, and Chlamydia.

Miller and Dorn (1990); Schakenraad and Dijkhuizen (1990); Miller *et al.* (1993) and Bennett *et al.* (1999) reported that Bovine mastitis is a highly prevalent disease in dairy cattle, and one of the most important diseases affecting the world's dairy industry and it places a heavy economic burden on milk producers all over the world.

Bansal *et al.* (1990); Kapur *et al.*(1992); Char *et al.*(1993); Lafi *et al.* (1994) described that *Escherichia coli* as the second most common etiological agent causing mastitis in cows following *Staphylococcus aureus*, among all the pathogens of bovine mastitis, *Staphylococcus aureus* is the predominant organism. Coagulase negative Staphylococcus (CNS) is also the prevalent bacterial pathogen in udder infections.

Hoblet *et al.* (1991) and Gruet *et al.* (2001) studied on bovine mastitis and found it to be the most costly disease to the dairy industry worldwide, with losses estimated at 2 billion dollars per year in the United States alone. These relevant economic losses are attributable to rejected milk, reduced milk quality, early culling, drug costs, veterinary expenses, and increased labor costs.

Holdway (1992); Capuco *et al.*(1992) observed that cows with subclinical mastitis are those with no visible changes in the appearance of the milk and/or the udder, but milk production decreases by 10 to 20% with undesirable effect on its constituents and nutritional value rendering it of low quality and unfit for processing. During milking, bacteria present near the opening of the teat find opportunity to enter the teat canal, causing trauma and damage to the keratin or mucous membranes lining the teat sinus.

Allore (1993); Harmon (1994) and Aller (1995) found that in India and Pakistan prevalence of sub-clinical mastitis is 17-93% in cows and 4-48% in buffaloes.

Mastitis is the most costly disease of the dairy industry all over the world. Mastitis in its subclinical form affect a major part of the cow's udder and the quality and quantity of milk reduced, so its detection in its subclinical form is very important.

Matsunaga *et al.* (1993); Aarestrup *et al.* (1995a) and Laevens *et al.* (1996) studied on other phenotypic characterization of *Staphylococcus aureus* which includes haemolytic activity, biochemical and enzymatic reactions, including activity on selective media, and antibiotic susceptibility patterns.

Pyorala and Pyorala (1994); Schukken *et al.*(1994) studied on intramammary infection sustained by *Staphylococcus aureus* which may result in clinical or subclinical mastitis and is usually associated with increased SCC. Appropriate treatment of mastitis during the lactation or dry period is an important component of any mastitis control program, but the outcome for treatment of mastitis caused by *Staphylococcus aureus* is variable and the probability of curing the disease is not high, primarily because of poor distribution of the drug in the inflamed udder and the occurrence of staphylococci resistant to antimicrobial agents During lactation, the cure rate of subclinical mastitis ranges widely, and this variability can be due to the choice of antimicrobial agent as well as to factors associated with the infected cow and the quarter. Therefore, cure is likely not a random event.

Pfutzner (1994); Wendt (1994); Blowey and Edmondson (1995) found a reason that these high percentages of culture-negative samples might be a low concentration of udder pathogens, e.g. *Escherichia coli*. Other pathogens such as mycoplasma, yeasts and moulds are difficult to cultivate. But these agents cannot be the explanation for all culture-negative milk samples from mastitis cows, because these agents are no common udder pathogens. Moreover there are no visible or palpable external changes, the infection is present and inflammation is occurred in the udder.

Muhammad *et al.* (1995) investigated that mastitis is the most dreadful disease confronting the dairy industry throughout the world but the situation in Pakistan is particularly very alarming and demands great attention for its control because of high economic losses due to this disease. According to him Mastitis is one of the limiting factors in the development of dairy industry in Pakistan. In addition to causing colossal economic losses to the farmers, the disease is important from consumer's and milk processor's point of view. This is because the milk from affected animal may harbor the organisms potentially pathogenic for humans (zoonosis) and processing of such milk results in suboptimal output of substandard finished fermented products like yogurt, cheese, etc.

Barmely *et al.*(1996) described that economic losses are due to (a) loss in milk production, (b) discarding abnormal milk and milk withheld from cows treated with antibiotics, (c) degrading of milk quality and price due to high bacterial or somatic cell count (SCC), (d) costs of drugs, (e) veterinary services and increased labor costs, (f) increased risk of subsequent mastitis, (g) herd replacement, and (h) problems related to antibiotics residues in milk and its products.

Miltenburg *et al.* (1996); Wedderkopp (1997); Barkema *et al.*(1998) said that these pathogens infect the udder generally through the ductus papillaris, which is the only opening of the udder to the outside world. Despite intensive aetiological research, still around 20–35% of clinical cases of bovine mastitis have an unknown aetiology i.e. 28% negative rate in 1045 cases of clinical mastitis. There are no pathogens in 35% of 6809 milk quarters in 3783 cows suffering from clinical mastitis. The percentage of culture-negative samples of both clinical and subclinical mastitis cases in the Netherlands has recently been determined to be approximately 25%.

Yalcin *et al.* (1999); Esslemont and Peeler (1993) and Schakenraad and Dijkhuizen (1990) observed that in Scottish dairy herds, facing high bulktank

SCC, the average annual costs of subclinical mastitis was 100 Pound Sterling/cow while in the UK and the Netherlands, the annual average revenue losses were calculated to be 42–84 Pound Sterling/cow and approximately 59 Euro/cow

Ott (1999) studied on subclinical mastitis which is considered the most economically important type of mastitis because of long term effects of chronic infections. Production losses due to subclinical mastitis on U.S. dairy farms were estimated to cost the dairy industry $1 billion dollars annually.

Hillerton (1999) observed that clinical mastitis is characterized by sudden onset, swelling, and redness of the udder, pain and reduced and altered milk secretion from the affected quarters. The milk may have clots, flakes or of watery in consistency and accompanied by fever, depression and anorexia. The sub clinical mastitis is characterized by having no visible signs either in the udder or in the milk, but the milk production decreases and the SCC increases, having greater impact in older lactating animals than in first lactation heifers. A negative relationship generally exists between SCC and the milk yield. Milk from normal uninfected quarters generally contain below 200,000 somatic cells /ml. A value of SCC above 300,000 is abnormal and an indication of inflammation in the udder. There is a plethora of evidence that the dairy cow milk has a natural level of 100,000-150,000 somatic cells/ml and higher SCC indicates secretory disturbance

Radostits *et al.* (2000) studied on etiology of mastitis and found that mastitis is mainly caused by *Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Corybacterium pyogenes, Streptococcus dysagalactiae* and *Streptococcus uberis etc.*

Radostits *et al.* (2000) examined that higher incidence *of Escherichia coli* mastitis may be due to poor hygienic conditions or intensive use of antimicrobials targeted against Gram positives for mastitis control. As a result *Coliform* mastitis remains as one of the most difficult diseases to treat in the modern dairy industry. Curative

therapy with antibiotics remains only moderately effective and depends on the stage at which the disease is treated. The most important factor in the control of *Escherichia coli* mastitis is the emergence of multiple drug resistant strains. Indiscriminate use of antibiotics in the treatment of mastitis has lead to the emergence of drug resistant strain

Sol *et al.* (2000) reported that antimicrobial therapy is a primary tool for controlling staphylococcal mastitis, and antimicrobial susceptibility tests can guide the veterinarian in selecting the most appropriate antimicrobial agent for treatment of IMI by *Staphylococcus aureus*. However, despite a variety of available antimicrobial agents, success in the treatment of *Staphylococcus aureus* mastitis, particularly during lactation, is still very low. In fact, *Staphylococcus aureus* pathogens have many characteristics that make them difficult targets for antimicrobial therapy.

Shoshani *et al.* (2000) studied that mastitis is of vital importance in its association with many zoonotic diseases in which milk acts as a vehicle of pathogens causing tuberculosis and brucellosis. Economic losses caused by this disease and found that it also posses the risk for the transmission of zoonotic diseases like tuberculosis, brucellosis, leptospirosis and streptococcal sore throat to human beings.

Phuektes *et al.* (2001) reported that negative cultures may also be due to bacteria inhibited by residual therapeutic antimicrobials or leukocytes. Environmental contaminants and intra-cisternal microorganisms can also represent a major problem in the interpretation of culture results. Moreover, microbiological culture of milk is time consuming. Species identification by standard biochemical methods requires more than 48 hrs for completing.

Bradley (2002); Vasudevan *et al.* (2003); observed that mastitis is the most frequent cause for antimicrobial therapy in cows and is being hard to eradicate

independently of a good antimicrobial susceptibility in vitro shown by the implicated microorganisms.

European countries standards (IDF, 1996) and Egyptian standards (Egyptian Standards, 2001) recommended that from public health view, the assessment of subclinical mastitis etiological pathogens aids to classify the healthy sound milk samples from those of public health hazards.

Fetrow (2000); Seleim *et al.* (2002) found mastitis to be the most frequent and costly disease of dairy cattle. According to him Losses due to mastitis can be attributed to both subclinical and clinical disease. Clinical mastitis losses are generally readily apparent and consist of discarded milk, transient reductions in milk yield and premature culling. Mastitis is one of the most costly and troublesome diseases in dairy cows in Egypt.

Lesile *et al.* (2002) El-Attar *et al.* (2002); Philpot (2003); Ullah (2004) examined that the invisible changes in subclinical mastitis can be recognized indirectly by several diagnostic methods including the California mastitis test (CMT), the Modified White Side test (MWT), SCC, pH test, chlorine and catalase tests. These tests are preferred to be screening tests for subclinical mastitis as they can be used easily, yielding rapid as well as satisfied results. CMT can detect more cases than MWT. Mastitis impairs the quality of milk and milk products.

Dingwell *et al.* (2003); Zdunczyk *et al.* (2003) studied that identifying and eliminating intramammary infection (IMI) may have significant economic benefits as preventing clinical mastitis and decreasing the amount of discarded milk. The prevalence of subclinical mastitis in dairy herds is often surprising to producers, moreover, sub-clinically infected udder quarters can develop clinical mastitis and the rate of new infections can be high.

Cai *et al.* (2003) studied on sub-clinically infected cows which are intermittent shedders of organisms and may cycle through low and high shedding patterns

during lactation. Milk culture may yield no bacteria from truly sub-clinically infected glands due to the presence of very low numbers of pathogens when samples are collected.

Vasudevan *et al.* (2003); Fox *et al.* (2005); Melchior *et al.* (2006b) concluded that mastitis staphylococci with biofilm forming ability effectively adhere and colonise in the mammary gland epithelium, and establish persistent infections. Biofilm impair the action of phagocytic cells from the host immune system and of antimicrobial compounds, and release planktonic cells from the outer layers, allowing the persistence of bacterial infections. Biofilm formation requires the bacterial attachment to solid surfaces promoted by surface proteins, the development of bacterial multilayers and their enclosing in a large exo-polysaccharide matrix.

Varshney and Naresh (2004); Saleh (2005); Moroni *et al.* (2006); Melchior *et al.* (2006b) reported that sub clinical mastitis is related to approximately 70% of economic losses of mastitis ,and found that susceptibility to mastitis is low in buffaloes when compared to cattle. Quarter-wise prevalence of intramammary infection in buffalo was 66%, especially during the peri-parturient period, whereas the incidence is highest during the 30 days after calving. Mastitis remains the most costly disease in dairy production, due to decrease milk production, increased health care costs and increased culling and death rates.

Thrusfield (2005) and Shakoor (2006) studied on the factors that determine the occurrence of mastitis. Among various microorganisms, bacteria (*Staphylococcus aureus and Streptococcus agalactiae*) have been reported to be the most commonly associated etiological agents of mastitis in dairy buffaloes and cows in Pakistan.

Jones (2006) studied that masses of neutrophils pass between the milk producing cells into the lumen of the alveoli, thus increasing the somatic cell counts and also

damaging the secretory cells. Increased number of leukocytes in milk causes increase in the number of somatic cells. Clots are formed by aggregation of leukocytes and blood clotting factors which may block the ducts and prevent complete milk removal, resulting in scar formation with proliferation of connective tissue elements. This results in a permanent loss of function of that portion of the gland. The milk ducts remain clogged, secretory cells revert to non-producing state, alveoli begin to shrink and are replaced by scar tissue. This helps in formation of small pockets making difficult for antibiotics to reach there and also prevents complete removal of milk.

Clutterbuck *et al.* (2007) studied on *Staphylococcus aureus* and *Staphylococcus epidermidis* which remain two of the most commonly isolated bacteria and able to form biofilms, highly organized multicellular complexes that represent an important virulence factor in staphylococci.

Chockalingam *et al*. (2007) observed that mastitis continues to be among the costliest diseases to the dairy industry, and annual economic losses attributed to this disease in the United States are estimated to approach $2 billion. Among cattle diseases, bovine mastitis is a serious problem which affects the basic income of the farmers depleting their dairy sources. Worldwide, mastitis is associated with economic losses of $35 billion annually. It adversely affects milk production whereby losses due to subclinical mastitis are more severe than those due to clinical cases. Controlling subclinical mastitis can reduce the losses in milk production substantially. Decreased milk production and quality, as well as veterinary expenses, all contributes to these economic losses

Almaw *et al.* (2008); Ahmed *et al.* (2008) and George *et al.* (2008) investigated that subclinical mastitis is 3–40 times more prevalent than its clinical counterpart and causes greater economic losses than clinical mastitis. Early diagnosis of mastitis is a must for reduction of production losses and for enhancing the

prospects of recovery. Also, the identification of sub-clinically infected gland is urgently required for successful control of mastitis in dairy animals. His study was designed to investigate subclinical mastitis in the main Egyptian dairy animals which are buffaloes that produce 65% of dairy product with special concept to find a practical marker for its early diagnosis.

Dhanabalan *et al*. (2008) observed that clinical and subclinical cases of mastitis are routinely treated with antimicrobials both intramammarily and parenterally. The use of antimicrobials over long periods has triggered the development of multidrug resistant strains, which has resulted in the use of increasing doses of antimicrobials, causing the danger of increasing amounts of drug residues in milk, a potential biohazard.

McDougall *et al.* (2009) studied that subclinical mastitis is a major problem affecting dairy animals all over the world as it causes enormous losses for breeders and consequently influences the national income of the country.

EFFECTS OF TEA EXTRACTS ON DIFFERENT MICROORGANISMS

Das *et al*. (1962); Yamamato *et al*. (1989); Toda *et al*. (1992); found that tea components also inhibit the growth of *Vibrio cholera* O1, *Streptococcus mutans*, *Shigella dysenteriae* and other bacteria grown in vitro.

Stagg *et al*. (1975); Ryu *et al*. (1982); Diker *et al*. (1991); Toda *et al*. (1989) and Hamilton-Miller (1995) stated that tea from the leaves of plant *Camellia sinensis* has been shown to have wide range of antioxidant, antiinflammatory, anti-carcinogenic and antibacterial activity against many pathogens.

Onishi *et al*. (1981); Toda *et al*. (1982); Kawamura *et al*. (1989); Sakanaka *et al*.(1989) showed that aqueous extracts of green tea inhibited cariogenic streptococci, including *Streptococcus mutans*. Extracts of tea inhibited and killed *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhi*, *Salmonella*

typhimurium, *Salmonella enteritidis*, *Shigella flexneri*, *Shigella dysenteriae*, and *Vibrio* spp., including *Vibrio cholerae*.

Okubo *et al*. (1989) observed that at a subcellular level, extracts of black and green tea inhibited the hemolytic activities of staphylococcal alphatoxin and the thermostable direct hemolysin of *Vibrio parahaemolyticus* against rabbit erythrocytes.

Hara *et al*. (1989); Ahn *et al*. (1990); Ahn *et al*. (1991) and Fukai *et al*. (1991) studied on tea extracts which have been found to be active against *Clostridium* spp. and phytopathogens such as *Erwinia* spp. and *Pseudomonas* spp. Further it was observed that *Staphylococcus aureus*, *Vibrio parahemolyticus*, Clostridium *perfringens*, *Bacillus cereus*, *Pleisomonas shigelloides* and *Aeromonas sobria* failed to grow in tea normally consumed by Japanese people.

Toda *et al*. (1989a; 1991) studied on the medicinal properties of *Camellia sinensis* L. ((Theaceae) which have long been recognized and there are reports proving its antibacterial nature against gram positive and gram negative bacteria. Tea at concentrations identical to those found in the beverage (a ‘‘cup’’ of tea contains ca. 3 mg of solids per ml) inhibited methicillin-resistant *Staphylococcus aureus*.

Nakane and Ono (1990) reported that EGC, EGCG and theaflavine-3,3’-gallate can inhibit RNA polymerase by competing with the nucleotide. ECG and EGCG, but not EC or EGC, are powerful antagonists of human immunodeficiency virus reverse transcriptase, causing 50% inhibition at concentrations of 10 to 20 ng/ml

Hattori *et al*. (1990); Fukai *et al*. (1991) reported that the activities of the theaflavins were similar to those of the simple catechins, thus casting doubt on the importance of the gallate moiety in the antimicrobial activity of black tea extracts. Furthermore studied on valuable anticariogenic effect of tea by the inhibition of the synthesis of insoluble glucans by *Streptococcus mutans*.

El-Gammal *et al*. (1986); Okubo *et al*. (1991) observed that the flavonols quercitin, kaempferol, and myricetin showed activity against gram-positive bacteria and phytopathogenic fungi in a screening test Quercitin had an MIC of 37 mg/ml for *Staphylococcus aureus* and was inactive against *Escherichia coli. Trichophyton mentagrophytes* and *Trichophyton rubrum*, but neither *Candida albicans* nor *Cryptococcus neoformans*, were inhibited by tea.

Laughton *et al*. (1991); Agarwal *et al*. (1992); Scott *et al*. (1993); Chung *et al*. (1999); Spencer *et al*. (2001); and Caturla *et al*. (2003) investigated on antioxidative and free radical scavenging activities of the polyphenolic components responsible for the antimicrobial activity of tea. These molecules have the capacity to modulate the physical structure of cell membrane by which a number of membrane dependent cellular processes, such as cell signaling and the cell cycle, arachidonic acid metabolism and cell proliferation, apoptosis, mitochondrial functionality may be influenced by the interaction of catechins with the cellular phospholipids palisade. It was examined that unfractionated extracts of both green and black tea possess modest antibacterial activity and that *Staphylococcus aureus* (MRSA), are being inhibited by the equivalent of a 1 :10 dilution of a cup of tea.The antimicrobial effects appears to be EGCG; in black tea extracts, the catechin dimmer theaflavin and its gallates are additionally responsible for antibacterial activity.

Shimamura and Hara (1992); Kubo *et al*. (1992); Horiuchi *et al*. (1992); Muroi *et al*. (1993) reported that tea polyphenols showed an important action on antimicrobial activity by combining with protein and inhibiting enzyme activity. Tea components are microbiologically active, but not at cup-of-tea concentrations. *Bordetella pertussis* is also inhibited by tea components. Combinations of the flavor compounds, especially indole with some of the sesquiterpenes, displayed marked bactericidal synergy.

Ikigai *et al*. (1993) showed that EC was much less active than EGCG. *Staphylococcus aureus* was more susceptible than *Escherichia coli*, consistent with a much greater binding of EGCG to staphylococci. The MICs of EGCG and EC were 73 and 573 mg/ml, respectively, for *Staphylococcus aureus* and 183 and 1,140 mg/ml, respectively, for *Escherichia coli*. The bactericidal effect of EGCG was attributed to membrane perturbation. Further study was done on susceptibility of bacterial strains to the tea extract which has been shown to be related to differences in cell wall components. EGCG concentration irreversibly damaged the bacterial cytoplasmic membrane on the basis that (PC) phosphatidyl choline liposomes leaked fluorescent dye following the exposure to the compound that is EGCG.

Spiro *et al*. (1995) reported that some of the important pathogen like *Listeria monocytogenes* and some some stain of *Escherichia coli* or *klebsiella pneumonia* were found to be resistance to aqueous extract of tea. Quantitative acess against *Candida albicans* and certain non-mycobacterial species like *M.kansai*, *M.pertuitum* were found to be resistant against tea extracts. At the concentration of 0.6mg/ml. crude extracts of tea was found to be effective against both antifungal and anti microbial agents.

Cooper *et al*. (2005); Qiang *et al*. (2006); Si *et al*. (2006) and Almajano *et al*. (2008) stated that tea polyphenols play an important role in protein precipitation, enzyme inhibition, antioxidation and have a broad anti- microbial spectrum inhibiting most food born pathogens, such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogens* and *Staphylococcus aureus*.

Mabe *et al*. (1996); Tsuchiya *et al*. (1996); Cox *et al*. (2001) studied on catechins partitioning in the lipid bilayer membrane which result in loss of cell structure and function and finally the cell death.

Yam *et al*. (1997, 1998) reported that aqueous extract of tea Camellia sinensis inhibited a wide range of pathogenic bacteria which include methicillin resistant *Staphalococci*, *Yersinia*, *Staphylococcus epidermidis* and other pathogenic microorganisms. Extracts of green tea have the capacity to reverse methicillin resistance in MRSA isolates at concentrations much lower than those needed to produce inhibition of bacterial growth.

Berger bachi and Tschierske (1998) studied on PBP2a which has a lower affinity for β-lactams than other PBPS in particular its analogs PBP2 and maintance transpeptidase activity in the presence of β-lactum concentration that saturate other PBPS.

Wilkilson (1997) and Hiramatasu *et al.* (1998) reported on the halotolerance in *Staphylococcus aureus* which can be modulated by treatment with green tea polyphenolic compound and advocated the use of tea extract for preservation of food stuffs when staphylococcal contamination is a problem.

Ehlert (1999) reported that B-lactam resistance in *Staphylococcus aureus* is associates with the pruence of a 40-60 kb chrosomal element (mec), that includes the mec A gene encoding an additional penicillin binding protein (PBP) termes PBP2a. the PBPs catalyse the insertion and cross linling of newly synthesized peptidoglycan precursors into the staphylococcal cell wall they are the targets for β-lactam antibiotics .

Chou *et al*. (1999) stated that differences in antimicrobial activities of tea have been found to be related with the kind and degrees of fermentation of tea. Green tea contains high concentrations of catechins such as (0)–epicatechin (EC), (0)-epigallocatechin (EGC), (0)-epicatechingallate (ECG) and (0)- epigallocatechin gallate (EGCg).

Hashimoto *et al*. (1999); Shiota *et al*. (1999) reported that catechin gallates bound more avidly than either EC or EGC to small unilamellar vesicles produced from

PC and ECg had a greater affinity for the bilayer than EGCg and stated their relative affinity in terms of partition co efficients in n-octanol saline. ECG and EGCG markedly lower the MIC of methicillin, oxacillin and other β-lactam antibiotics in clinical isolates of MRSA.

Karakaya and Kavas (1999); Lau *et al*. (2002); Frei and Higdon (2003); Rietveld and Wiseman(2003); Chattopadhyay *et al*. (2004); Wheeller and Wheeler (2004) and Nihal *et al*. (2005) reported that fresh green tea leaves are rich in monomeric flavanols, known as catechins and (-) epigallocatechin gallate (EGCG) is the most abundant green tea catechins. Catechins are present at leaves of 30-40% of the dry weight of fresh green tea leaves.

Hashimoto *et al*. (1999); Hamilton-Miller and Shah (1999 , 2000); Kumazawa *et al*. (2004); Stapleton *et al*. (2005) found that catechins in the nanomole range are able to modulate the structure and function of model membranes due to their capacity to partition in to the phospholipids palisade. Aqueous extract of green tea are ECg which increase the cell wall thickness and stimulate the formation of pseudo multicellular aggregates. Antibacterial activity was attributable to the catechin gallates, with ECg showing greater potency than either EGCg or Cg.

Sakanaka *et al*. (2000) reported that the inhibitory action of tea polyphenols towards the development and growth of bacterial spores of *Bacillus* bacteria, tea polyphenols showed antibacterial effects towards *Bacillus stearothermophilus*, which is a thermophilic spore-forming bacterium. The heat resistance of *Bacillus stearothermophilus* spores was reduced by the addition of tea polyphenols. *Clostridium thermoaceticum*, an anaerobic spore-forming bacterium was reduced heat resistance of its spores in the presence of tea polyphenols. Epigallocatechin gallate, which is the main component of tea polyphenols, showed strong activity against both *Bacillus stearothermophilus* and *Clostridium thermoaceticum*. The

heat resistance of these bacterial spores was more rapidly decreased by the addition of tea polyphenols at high temperatures.

Lakenbrink (2000) conducted the research on the polyphenolic, flavonoid, and caffeine compositions of four commercial tea bag products (typical of those used in the UK, US, continental Europe, and the Middle East) and beverages prepared from them under a range of typical consumer use conditions have been studied. Leaf composition was determined by extraction with aqueous methanol .The absolute compositions of all four products were remarkably similar in terms of most phenolic compounds. The flavonoids comprised the major proportion (93-94%) of the total phenolics estimated by the Folin- Ciocalteu method.

Hamilton Miller (2001); Isogai *et al*. (2001) stated that various components of *Camellia sinensis* either in the form of green or Black Tea are simple catechins, which have anti-cariogenic properties in vitro. These include a direct bactericidal effect against *Streptococcus mutans* and *Streptococcus sobrinus*; prevented the bacterial adherence to teeth; inhibition of glucosyl transferase, thus limiting the biosynthesis of sticky glucan and inhibition of human and bacterial amylases. There is synergy between green tea extract and levofloxacin against enteroahaemorrhagic *Escherichia coli*.

Kim *et al*. (2001) concluded the antibacterial potential of the methanolic extracts petals of *Camellia japonica* L., against food borne pathogens in microbiological media and food. The extract showed good bactericidal response against the pathogens *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus*. Acidic, basic and neutral methanol extract fractions of *Camellia japonica* which inhibited the growth of food borne pathogens in microbiological media and food

Kajiya *et al*. (2001) reported that catechins with epi (cis) stereochemistry partitioned in to pc liposomes more rapidely that they are non-epi(trans)

configured counterparts and also in epicatechins such as ECg and EGCg the hydrophophic domain in the region of the ester bond and C-ring is relatively exposed and liable to perturb the bilayer .

Frei and Hington (2003); Neuhaus and Baddiley, (2003) reported that the antimicrobial activity of tea in cell and cell membrane aim to scavenge free radicals. They further stated that tea catechins have antioxidant action and belived to scavange free radicals, there by inhibiting oxidation. This mechanism is also self explanatory for antimicrobial activity in cell and cell membranes. ECg polymer get anchored into the staphylococci cytoplasmic membrane and modulate the sensitivity of MRSA to β-lactum antibiotics.

Caturla *et al*. (2003); Frei and Hington (2003); Karakaya and Kavas (1999); Nihal *et al*., (2005) stated that the most important beneficial effects of tea is the antioxidant activity and free radical-scavenging ability of the polyphenol components of tea leaves. Membrane interaction of catechins depends on degree of hydroxylation of the β-ring, the presence of a gallate moiety and stereochemistry of the C-ring. Catechin gallates like ECg penetrate in to the hydrophobic core of the membrane were as EC and EGC adopt a more superficial location close to the phospholipids water interface.

Campos, Couto and Hogg (2003); Negi, Jayee prakasha and Jena (2003); Taguri, Tanalea and Kouno, (2004) investigated that the tolerance of bacteria to polyphenols depends on the bacterial species and the polyphenol structure. Tea extracts act as inhibitor of food pathogen including *Staphylococcus aureus* , *Shigella dysenteria*, *Vibrio cholera*, *Campylobacter jejuni*, *Listeria monocytogenes*, etc.

Arakawa *et al*. (2004); Roccaro *et al*. (2004); Zhang and Rock (2004) noted that EGCg inhibited the FabG anf FabI reductase steps in the fatty acid elongation cycle and hence the antibacterial action is due to the capacity of EGCg to interfere

with these NAD (P) dependent intracellular procuses as EGCg and other catechins donot gain entry in to cells but exert their effects from the cell membrane. Cytoplasmic membrane damage results from the generation of hydrogen peroxide by EGCg with in the bilayer. EGCg increases the accumulation of tetracycline in *Staphylococcus aureus* strains by inhibiting the Tet (K) and Tet (B) efflux pumps. EGCg also enhances the activity of norfloxacin against a Nor A harboring *Staphylococcus aureus* strain.

Stapleton *et al*. (2004a, 2005) examined the capacity of a range of catechins and gallates to reverse β-lactam resistance in clinical isolates of MRSA and found that 12.5-25mg/l of ECg reduced MIC values for oxcacillin from levels of 256 and 512 mg/l to 1-4mg/l. this modification from full resistance to below the antibiotic break point at which drugs are deemed to be of therapeutic value raises the possibility that ECg are stable catechin gallate analogues could be used in combination with suitable β-lactam agents to treat MRSA infections. It was also determined that that gallate moiety of ECg was essential for oxacillin-modulating activity, although gallic acid did not increase the sensitivity of MRSA to β-lactam agents. ECg effect the cell wall autolysis production and activity which are involved in cell wall turn over and cell separation. They further reported that ECg stimulated the release of lipoteichoic acid from the cell wall.

Tiwari *et al*. (2005) studied that the synergistic antimicrobial activity of tea and antibiotics against enteropathogens. Antimicrobial activity of boiled water tea extract and organic solvent extract were studied against *Salmonella typhimurium* 1402/84, *Salmonella typhi*, *Salmonella typhi* Ty2a, *Shigella dysenteriae*, *Yersinia enterocolitica* C770, and *Escherichia coli* determining minimum inhibitory concentration, minimum bactericidal concentration and death rate kinetics at MBC of tea extract in presence of sub inhibitory concentration of antibiotic. Black Tea extracts effectively inhibited the growth of *Salmonella typhimurium* 1402/84, *Salmonella typhi*, *Salmonella typhi* Ty2a, *Shigellae dysenteriae dysenteriae*,

Yersinia enterocolitica C770, and *Escherichia coli*. Based on death rate kinetics results, *Salmonella typhi* Ty2a appeared to be highly sensitive and *Yersinia enterocolitica* C770 the most resistant. Chloramphenicol and tea extract in combination inhibited the growth of *Shigellae dysenteriae* at 2.5 µg/ml chloramphenicol (MIC 5 µg/ml) and 5.094 mg/ml Black Tea extract (MIC 9.089 mg/ml). Tea extract showed synergistic activity with chloramphenicol and other antibiotics like gentamycin, methicillin and nalidixic acid against test strains.

Anderson *et al*. (2005b) reported that modification to the β-ring of ECg affected the degree of resistance modulation, in addition to functional differences between ECg and EGCg, which differ only in the degree of hydroxylation of the β-ring unnatural mono and di hydroxyl analogues of ECg were able to fully sensitise MRSA strains to oxacillin.

Friedman (2007) studied that the tea leaves produce organic compounds that may be involved in the defense of the plants against invading pathogens including insects, bacteria, fungi, and viruses. These metabolites include polyphenolic compounds, the six so-called catechins, and the methyl-xanthine alkaloids caffeine, theobromine, and theophylline. These substances impart the black color to Black Tea.

Chikara *et al*. (2008) reported that antimicrobial activity of EGCG on anti-Listeria monocytogenes was due to inhibition of hemolytic and cholesterol binding activity of listeriolysin O, which distrupts the phagosomal membrane during the escape of *Listeria monocytogenes* .

Taylor *et al*. (2009) concluded that non-galloyl catechins bind superficially to the outer surface of the membrane and helps in penetration causing increase in lipid order, producing tightly packed and extended acyl chains in the bilayer, also reduces the efficiency of function of many membrane proteins such as the PBPs, resulting in changes to the cell wall structures and also affect the traffic of proteins

across the membrane. Penetration of catachin gallates in to the bilayer displaces lipoteichoic acid, a macromolecule which modulate the sensitivity of MRSA to β-lactum antibiotics.

III. MATERIALS AND METHODS

The present study was undertaken to evaluate the antimicrobial properties of tea (*Camellia sinensis*) on bacterial mastitic isolates. The laboratory work for the experiments was carried out in the Department of Veterinary Microbiology, in the College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar. , Animal Disease Research Institute (A.D.R.I.), Phulnakhara were also used for preliminary testing of milk for mastitis. Milk Samples were received from Teaching Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Bhubaneswar, Animal Disease Research Institute (A.D.R.I.), Phulnakhara, and from different organised dairy farms. The work was undertaken for March-2011 to June-2011(Summer season).

Technique for Collection of milk for bacteriological test

Milk samples were collected aseptically from the affected quarters found positive for mastitis through Modified California Mastitis Test. The udder of the affected cow was cleaned with lifebuoy soap and water, wiped with clean cloth socked in 0.1 % potassium permanganate solution and allowed to dry under normal atmospheric temperature. The teat orifice was cleaned with 70 % alcohol by averting them with finger pressure. About 10 ml of milk sample was collected in a sterilized test tube after discarding 4-5 strips of milk and was kept in refrigerator in the same day for different laboratory test.

The California Mastitis Test (C.M.T.) was employed at the door side of the farms for securitization of sub-clinical mastitis. The clinical mastitis have been detected in the Bacteriological laboratories as described in the table. Physical examinations were also conducted on all CMT-positive cows. Microbiological examinations were carried out in all mastitic milk samples.

Preparation of MCMT reagent :

Modified California Mastitis Test reagent was prepared as per the formulation of Pandit and Mehta(1969).

Sodium Lauryl sulphate 3%	- 100gm
Bromocresol purple 0.5%	- 2ml
Distilled water	- 1000ml
pH adjusted to 8.2	

Bromocresol purple 0.5%- 2ml was added to Sodium Lauryl sulphate 3 %-100gm in 1000 ml distl.water. The pH of the solution was adjusted to 8.2 with the help of a pH meter using N/ 10 HCL or NaOH. The final solution was purple in colour. Stock solution of the reagent was stored at room temperature in an amber coloured glass stopper bottle, properly labeled, and was used up to three months after which fresh test reagent was once again prepared.

Test Procedure for MCMT

For the purpose of conducting Modified California Mastitis Test, a four chambered plastic paddle was used along with the MCMT solution. The chambers of the Paddle were marked Right Fore (RF), Right Hind (RH), Left Fore (LF) and Left Hind (LH) in order to avoid confusion. About 3 ml of milk from each teat was striped directly into the respective ear mark chamber after discharging 4-5 strips of milk, if the test is to be carried out in the Cow shed. If it is to be tested in laboratory, the milk is to be brought in sterilized vials of 5ml size with screw cap or in sterilized test tube, to the Lab. Labeling as RF,RH, LH, LF on the vials. First poured 3ml of milk in four respective chambers of the Paddle and added with equal quantity of MCMT reagent into the paddle. The Paddle was rotated by keeping it parallel to a horizontal plane clockwise or anticlockwise so that the milk

got thoroughly mixed with the reagent. Formation of mild to distinct gel in the respective chambers within 15-20 seconds was considered as positive results for mastitis and recorded as per the degree of reaction as follows:

Classification of MCMT reaction with milk samples

Sl. No.	Reaction observed	Interference
1	Colour of the reagent remains unchanged without any precipitation and formation of gel.	Negative (-)
2	Slight slime formation noticed with rocking of paddle from side to side.	Trace (+)
3	Formation of distinct slime without formation of gel which may disappear in course of time.	Moderately Positive (+ +)
4	Colour turns to greenish with the formation of gel.	Fairy Positive (+ + +)
5	Distinct slime formation occurs immediately after mixing the solution. When the paddle swirls the surface of the solution becomes convex due to increased viscosity.	Highly Positive (+ + + +)

ISOLATION OF CAUSATIVE ORGANISM

Brain heart Infusion broth, Mueller Hinton Agar, Blood agar plate (five to ten percent Sheep blood), EMB agar, Mac Conkey's agar were employed for isolation of bacteria (Buxton and Fraser, 1977). The BHI broth, Mueller Hinton agar, Mac Conkey's agar , were prepared by rehydrating the dehydrated powdered media, of

Dehydrated HiMedia, as per instructions labeled by manufacturer and required quantities of blood / antibiotics were added subsequently. The plates, slants and broth were kept in incubator at 37 ^{o}C for 24 hours for sterility test. Petriplates with media were dried as described by Crickshank *et al.* (1980) before streaking.

One loopful each of sediment from centrifuged milk was inoculated in BHI broth , streaked on Muller Hilton agar and blood agar plates.. Single colonies on blood agar plate were examined and their haemolytic pattern was studied. Smears were made from the suspected colonies from blood agar plate, Muller Hilton plate as well as from BHI broth culture and stained by Gram's stain (Merchant and Packer, 1967) and a tentative identification was made. Any isolate appearing diagnostic was stored in pure culture at 4 ^{0}C to 8 ^{o}C for further tests.

BACTERIAL IDENTIFICATION PROCEDURES

The Petri plates with the media , after drying for one hour in a bacteriological incubator at 37 ^{o}C , were streaked with one loopful of milk/broth culture across the surface of the solid medium in parallel lines and serial dilution was done. The organisms were grown in isolated colonies along the ends of the last few streaks (Wilson and Miles, 1965).

For the isolation of *Staphylococci*, Specific media Manitol Salt Agar was used. As with blood agar, Sterilized skimmed milk after autoclaving was mixed agar cooled to 56 ^{0}C. Crystal violet blood agar was prepared for the isolation of *Streptococci* by adding sheep blood and crystal violet to melted Mueller Hinton agar at 560C in final concentration of 0.0002 per cent. For *Streptococci* isolation, Edwards` Modified Blood Agar was used.(Buxton and Fraser,1977)

For the isolation and identification of *Enterobacteriacae* the methods outlined by Edwards and Ewing (1972) were followed. In order to isolate *Escherichia coli*, EMB agar were streaked from milk directly and incubated for 24 to 72 hours. Both lactose fermenting and non-lactose fermenting individual

colonies were fixed out separately in pure culture for further examination. For *Pseudomonas spp*. Pseudomonas Isolation agar was used .

Biochemical tests

The different biochemical tests performed and their techniques are as follows:

(a) Indole test : This test was performed as the lines of Cruickshank *et.al*. Op. cit.

(b) Methyl Red and Voges Proskauer test : This test was performed as described by Cruickshank *et.al.* , Op. cit.

(c) H2S production test : The pure colony was inoculated in peptone water (Hi-Media) and incubated for 4 to 5 days with strips of dried lead acetate paper inserted between the plug and side of the test tube and observed daily up to seven days for the blackening of the paper strip due to the production of hydrogen sulphide (Cruickshank *et.al.* , Op.cit).

(d) Citrate Utilisation test : Simmon's citrate agar slants (Hi-Media) were streaked with a loopful of pure culture and incubated for 96 hours. Positive reaction, if any, was indicated by the change in colour of the medium to blue with the growth of bacteria and negative finding was evidenced by no growth with the original greenish colour of the medium remaining unaltered.

Other tests performed such as

(e) Haemolysis : Blood agar plates, incubated aerobically at 37 ^{0}C , were examined after 24 hours for the presence of haemolytic zones. Negative plates were held in the refrigerator overnight and also at room temperature for 18 hours before declaring any strain as non-haemolytic.

(f) Coagulase test : This was performed as described by Merchant and Packer (Op. cit.) . A loopful of agar plate culture was mixed with one ml. of fresh, citrated

rabbit plasma diluted 1:5 in broth and incubated at 37 ^{0}C. Cultures showing clot reaction, within three hours, were considered positive.

(g) Motility test : Motility was checked by hanging drop preparation from 18 hours old broth culture.

(h) Sugar fermentation test : The pattern of sugar fermentation was checked by using the carbohydrates like arabinose, glucose, sucrose. maltose, lactose, ramnose, raffinose, mannose, xylose, mannitol, dulcitol and salicin in this investigation. Peptone water with Andrade's indicator was used as the basal medium which contained one per cent of the substrate.

ANTIBIOTIC SENSITIVITY TEST OF SAMPLES SHOWING BACTERIAL GROWTH

The milk samples from cows showing bacterial growth in Brain heart Infusion broth were subjected to antibiotic sensitivity test before isolation and identification of bacterial species for providing treatment without delay. Antibiotic sensitivity of individual pure isolates were also performed subsequently to asses the changing pattern of sensitivity.

For the purpose of conducting the test, a sterile disposable swab was soaked with the liquid culture aseptically before flame and smeared into the surface of Mueller Hinton Agar plates. The plates were incubated for 30 minutes for drying of the smear. Antimicrobial sensitivity disc as described below and manufactured by M/S HI-Media lab. Pvt. Ltd. , were then placed at equal intervals and pressed with sterile forceps for proper fixing. The plates were then incubated at 37 ^{0}C for 24 hours and then sensitivity was recorded as per zone of inhibition suggested by Bauer (1982). The sensitivity was graded as "Highly sensitive", "Moderately sensitive", and "Resistant" as per the measurement of zone of inhibition suggested by the manufacturer on the basis of Kriby-Baur , interpretative chart.

ANTIBIOTICS USED FOR THE STUDY OF BACTERIAL SENSITIVITY

Sl.No.	**Antimicrobial discs used**	**Strength per disc**
1	Ampicillin (A)	10mcg
2	Amoxycillin (Ac)	30mcg
3	Ampicillin with Cloxacillin	10mcg
4	Amikacin (Ak)	30 mcg
5	Azithromycin (At)	15 mcg
6	Cloxacillin (Cx)	30mcg
7	Ciprofloxacin (Cf)	5 mcg
8	Ceftriaxone (Ci)	30 mcg
9	Cephotaxime (Ce)	30 mcg
10	Ceftazidime (Ca)	30 mcg
11	Cefixime (Cfx)	5 mcg
12	Cefdinir (Cdn)	5 mcg
13	Cefalexin (Cp)	30mcg
14	Cefadroxyl (Cq)	30mcg
15	Cefuroxime Sodium (Cu)	30mcg
16	Cefacter (Cj)	30mcg
17	Clarithromycin (Cw 0	15 mcg
18	Chloromphenicol (C)	30 mcg

19	Enrofloxacin (Ex)	10mcg
20	Furoxone (Fx)	50mcg
21	Gentamicin (G)	10 mcg
22	Gatifloxacin (Gf)	5 mcg
23	Levofloxacin (Le)	5 mcg
24	Lomefloxacin (Lo)	10 mcg
25	Moxifloxacin (Mo)	5 mcg
26	Neomycin (N)	30 mcg
27	Ofloxacin (Of)	5 mcg
28	Pifloxacin (Pf)	5mcg
29	Penicillin-G (P)	10 units
30	Piperacillin with Tazobactam (Pt)	100 mcg
31	Streptomycin (S)	10 mcg
32	Sparfloxacin (Sc)	5 mcg
33	Sismomycin (Ss)	10mcg
34	Tobramycin (Tb)	10 mcg
35	Tetracycline (T)	30 mcg

MATERIALS USED FOR ESTIMATION OF PARAMETERS

1. MCMT reagent
2. Plastic Paddle
3. Sterilized test tubes of 10 ml
4. Screw cap sterilized vials-5ml
5. 70% alcohol
6. 1% Potassium permanganate

7. BHI broth
8. MHA agar
9. Blood agar
10. Thermocool Box
11. Incubator
12. Hot air Oven
13. Auotoclave machine
14. Laminar flow
15. Antimicrobials discs
16. Biochemical reagents
17. Different sugar solutions
18. Gram’s Staining ,
19. Sugar tubes
20. Microscopic glass slides
21. Compound Microscope
22. Bunsen burner
23. Petri plates
24. Aluminum foil
25. Aluminum loop
26. Test tube stand

27. Conical flasks of diff. size.
28. Glass beads.
29. Glass slides
30. Electrical balance
31. Peptone water
32. Sheep blood
33. Rabbit plasma.
34. Microwave extractor
35. Sterile disc
36. Ethanol (90%)
37. Methanol (100%)
38. Lipton brand tea

Selective Media

39. Manitol Salt agar
40. Pseudomonas Isolation Agar
41. Nutrient Agar
42. Mac Conkey Agar
43. EMB Agar
44. Simmond Citrate Agar

METHODS OF EXTRACTION OF TEA

Tea samples:

Tea samples used in the present study was collected from departmental store of Bhubaneswar, Orissa. All tea samples are of Lipton brand. The botanical name of tea is *Camellia sinensis* Linn; family Theaceae and the part of plant used were the Leaves.

Preparation of Crude Extracts

Solvent extraction

100 grams of dried tea powder was extracted with 200 ml of methanol kept on a rotary shaker for 24 hr. Thereafter, it was filtered and centrifuged at 5000 g for 15 min. The supernatant was collected and the solvent was evaporated to make the final volume one-fifth of the original volume (Sasikumar *et al*., 2005). It was stored at 4^{o}C in airtight bottles for further studies.

Aqueous extraction

100 grams of tea powder was extracted in distilled water for 6 hr at slow heat. Every 2 hr it was filtered through 8 layers of muslin cloth and centrifuged at 5000 g for 15 min. The supernatant was collected. This procedure was repeated twice and after 6 hr the supernatant was concentrated to make the final volume one-fifth of the original volume (Sasikumar *et al*., 2005)

Procedure of aqueous extraction of tea sample:

The tea extracts are prepared as per the method of Esimone *et al*. (2003) with slight modification. Ten grams of the commercial Lipton brand tea sample were weighed and boiled in 50 ml of water for 5 min. The suspension was filtered and evaporated to dry in an evaporating dish over a hot water bath maintained at temperature 69 ^{o}C. The required amount of tea extract was weighed out and

dissolved in the required amount of water to obtain the desired concentration of tea extract solution. This method was followed both for green and black tea.

Procedure of methanolic and ethanolic extraction of tea samples:

The purchased tea samples (Green tea and Black Tea) was grinded to obtain fine powder. The sample powder of both types of tea samples were triturated with redistilled methanol and ethanol for 4-6 days by Soxhlet apparatus and process was repeated three times to get desired amount of tea extracts. The solvent was removed in the rotary evaporator to yield the crude methanolic and ethanolic extracts stored at 4 ^{o}C.

The extracts of all tea samples were diluted with solvent (Ethanol and Methanol) and with combinations of different antibiotic (Chloramphenicol, Tetracycline, Levofloxacin and Gentamicin) with different concentration (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mg/ml) for sensitivity test of different bacterial mastitogenic isolates.

Preparation of tea extracts by using Multi wave digestion system:

According to the methods of (Pan *et al*. 2003) the green tea extract (Lipton brand) was prepared by Multi-wave 3000 (Anton Par) digestion system by taking 2 gm of finely grinded powdered tea leaves and 20 ml of methanol in each vessel. The organic solvent, methanol was used for extraction of polyphenols from the green tea powder. Extraction was done in closed system by Anton Par-Multiwave 3000-801-V of Microwave Assisted Extraction System at 80^0 C for 25 minutes followed by 15 minutes cooling. The process of extraction continued for 25 minutes at 80^0 C temperatures. The green tea extracts were filtered through Whatman No.1 filter paper before use. Both the extracts were exposed to a temperature of 50^0 C at 200 hpa pressure in rotary evaporator to obtain a concentrated extract of green tea in methanol. This process was repeated for green tea with ethanol and water as solvents. The procedure of extraction remains the same for ethanol and water as

solvent like it was followed for methanol as solvent. Then this above procedure was repeated for black tea powder with the same three types of solvents i.e. methanol, ethanol and water. The amount of tea leaves, the amount of solvent taken, the temperature, pressure, and time of extraction all parameters remain same during the extraction process. Then after getting required amount of extracts for use the extracts are stored immediately in refrigerator at 4^0C temperature in sterile 15 ml vials for use within 24 hr.

Preparation of antibiotic and tea discs

The detail procedure of Esimone *et al*. (2003) was followed with slight modification. According to the procedure circular filter paper discs (6 mm diameter) were prepared with the aid of an office paper perforator. The discs were placed in a Petri dish and sterilized in an autoclave. Dilutions of several concentrations of the tea and a suitable antibiotic were made in test-tube using sterile water. The paper discs were then aseptically transferred into the tubes containing the drugs solutions and allowed to absorb the solutions for about 15 s. The discs were then aseptically transferred to empty sterile test tubes and allowed to air dry while the mouth of the test tubes is still plugged with cotton wool. Each of the test tubes containing the dried discs was labeled with the strength of the solution of drug in which the paper discs were dipped. It had been established that the 6 mm disc has a capacity of absorbing 10 µl of drug solution at saturation.

Exactly 20 ml of molten nutrient agar in sterile Petri dish was seeded with 40 µl of the standardized test microorganism, well distributed and allowed to set on a horizontal plane. Discs containing antibiotic and tea extracts soaked in solution of 50 mg/ml (equivalent to 500 µg/disc) were aseptically placed on the seeded nutrient agar plates. This procedure was performed in 4 replicate plates for each test microorganism and also for the combination of the different antibiotics and different tea extracts. After a 30 min pre-diffusion time interval, the plates were

incubated at 39°C for 24 h. Thereafter, the diameters of zones of inhibition surrounding the discs were accurately measured and their relative susceptibility pattern deduced. This method was followed both for green and black tea samples and for all types of tea extracts i.e. (aqueous, methanolic and ethanolic).

METHODS OF CALCULATION OF MINIMUM INHIBITORY CONCENTRATION OF TEA EXTRACTS BY DISC DIFFUSION METHOD

The antibacterial assay of aqueous and methanolic extracts was performed by Bauer *et al*. (1996).The Muller Hinton Agar media, along with the inoculums (10^6 cfu/ml) was poured into the petri dishes. For the agar disc diffusion method, the disc (0.7 cm) (Hi-Media) was saturated with 100 mg/ml of the test compound, allowed to dry and then placed on the upper layer of the seeded agar plate. The plates were incubated overnight at 37 ^{0}C. Antibacterial activity was determined by measuring the diameter of the zone of inhibition (mm) surrounding bacterial growth. For each bacterial strain, controls were included that comprised pure solvents instead of the extract (Ayyappa *et al*. , 2009).

Disk diffusion method which is the method of choice for rapid sensitivity of a number of isolates to a range of antimicrobials was followed. For the disk diffusion assay 1 ml of each bacterial suspension was uniformly spread on a solid growth medium in a Petri dish. Four sterile paper disks (6 mm in diameter) were placed on the surface of each agar plate and were impregnated with 10 µl of the diluted tea extract. Plates were incubated for 24 hrs under appropriate cultivation conditions. Antibacterial activity as MIC was determined as the lowest concentration of different types of tea extract which produced an inhibition zone around a disk followed by the 24-hrs incubation according to the methods of (Valgas et al., 2007). Disks impregnated with sterile distilled water and methanol served as negative controls and a disk with an antibiotic (Tetracycline,

Levofloxacin , Gentamicin and Chloramphenicol, Hi Media) served as a positive control. Replicas at each concentration of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 mg/ml were performed for green tea and black tea samples and for three different types of extracts i.e. aqueous, methanolic and ethanolic. The test bacteria used for the the above experiment are *Staphylococcus spp., Streptococcus spp., Escherichia coli, Pseudomonas spp., Klebsiella spp and proteus spp.*

Inoculum size commonly selected according to the methods of (Smith et al., 1994) is 10^6 cfu/ml. The inoculum preparation was started with a selection of a defined number of colonies in order to achieve turbidity that is known to produce a semi-confluent growth. This semi-quantitative way of inoculum presentation results in big variations so the adjustment of suspension's turbidity is being performed using a McFarland 0.5 barium sulphate standard. Further dilution of the suspension was done before it is applied to the agar surface. It was noted that bacterial species differ in the number of cells or colony-forming units per milliliter required to achieve semi-confluent growth. Cultures of equal turbidity when diluted in fixed proportions do not necessarily result in a semi-confluent growth. The culture media used is Mueller Hinton agar to provide standard reproducible environment for optimum bacterial growth without affecting the antibiotic activity against the test organism. Firm contact between the agar medium and the antimicrobial agent disk was ensured. The inoculum is prepared either by colony suspension in a broth medium and used as working suspension or by broth inoculation with one or two colonies followed by 4–6 hr incubation and inoculum preparation from the broth. Techniques of inoculum production was followed according to McFarland standard like wet swab method. The lowest concentration of the antimicrobial treatment that inhibited visible growth of the pathogens after incubation was taken as the MIC of the tea extracts (Anand bhaskaran et al., 2009).

McFarland standard: a number of colonies were picked up and a suspension was made and cfu/ml was adjusted by visual comparison to a known McFarland

standard. A predetermined volume of the adjusted suspension was placed on the petri dish was spread evenly on the surface.

Dry swab: a number of colonies were picked up and an approximate suspension was made in a known volume. A loop full volume of the suspension was spread in all the plate using a loop.

Wet swab: a number of colonies were picked up and an approximate suspension was made in a known volume. Dilutions were performed if necessary. A sterile cotton swab was submersed in the solution and was then used for spreading a quantity on the agar surface.

Flooding method: a number of colonies were picked up and an approximate suspension was made in a known volume. Dilutions were performed. A known volume of the suspension was removed using a volumetric tube and was then spread on the agar surface. After a tilting circular movement, excess liquid was removed and plates are left to dry.

The lowest concentration of the antimicrobial treatment that inhibited visible growth of the pathogen after incubation was taken as the MIC of the treatment. The lowest concentration of the treatment that prevented growth of the organism after subculture on Mueller Hinton Agar followed by dilution and plating was taken as the MBC. Triplicate samples were included for each treatment, and the experiment was performed 3 times.

Table-1 : Sources of milk samples collection

Name of institution/dairy farm from where milk samples collected	Number of milk samples collected	Percentage of milk samples collected from different institute/dairy farm
1.Teaching Veterinary complex, C.V.Sc.,Bhubaneswar	15	15.6
2.C.I.L.,Cuttack	4	4.16
3.Animal Disease Diagnosis Institute ,Phulnakhara	37	38.54
4.Prema Dairy farm,Laxmisagar,Bhubaneswar	7	7.29
5.Ranjan Dairy farm,Patrapada,Bhubaneswar	9	9.37
6.Veterinary Microbiology Department,O.V.C.,Bhubaneswar	17	17.70
7.Instructional Livestock farm,O.U.A.T.,Bhubaneswar	7	7.29
Total	96	100

Table No.2: Biochemical tests of bacterial isolates isolated from bovine mastitis milk.

Sl. no	Biochemical Tests	Staph. Aureus	Staph epider midis.	Strepto coccus	E.coli	Pseudomonas aeruginosa	Corynebacteri um	Klebsiella spp	Bacillus spp.	Proteus spp.
1	**Indole**	-	-	ND	+	-	-	-	-	+
2	**M.R**	+	+	ND	+	-	-	-	-	+
3	**V.P**	+	+	ND	-	-	-	+	+	-
4.	**Catalase**	+	+	-	+	+	+	+	+	+
5.	**Oxidase**	-	-	ND	-	+	-	-	-	-
6.	**Nitrate reduction**	+	+	ND	+	-	-	+	+	+
7.	**Citrate utilization**	-	-	ND	-	+	-	+	+	-
8.	**H2S Produciton**	-	-	ND	-	-	-	-	-	+
9.	**Gelatin liquefaction**	+	+	-	-	+	-	-	+	+
10.	**Urease**	-	-	NA	-	+	+	+	-	+
11.	**Aesculin hydrolysis**	-	-	-	ND	ND	ND	ND	ND	ND
12.	**Ammonia from arginine**	ND	ND	+	ND	ND	ND	ND	ND	ND
13.	**Hipurate Hydrolysis**	+	+	+	ND	ND	ND	ND	ND	ND

Table 3: Sugar fermentation tests of bacterial isolates obtained from bovine mastitic milk.

Sl. No.	Sugar test	Staph. Aureus	Staph epider midis.	Strepto coccus	e.coli	Pseudomonas aeruginosa	Corynebacterium	Klebsiella spp.	Bacillus spp.	Proteus spp.
1.	**Glucose**	A	+	+	AG	-	+	AG	A	+
2.	**Sucrose**	A	+	+	AG	-		AG	+	+
3.	**Galactose**	+	+	NA	ND	-	-	ND	ND	ND
4.	**Mannitol**	A	-	+	AG	-	-	+	-	+
5.	**Salicin**	-	-	+	-	-	-	AG	AG	-
6.	**Glycerol**	+	+	+	ND	-	ND	ND	ND	ND
7.	**Maltose**	A	+	+	AG	-	+	ND	ND	+
8.	**Trehalose**	ND	+	+	ND	-	ND	ND	ND	ND
9.	**Raffinose**	-	-	+	ND	-	ND	ND	ND	ND
10.	**Lactose**	A	+	+	AG	-	+	AG	A	+
11.	**Mannose**	A	+	ND	ND	-	ND	ND	ND	ND
12.	**Sorbitol**	-	-	+	+	-	ND	ND	ND	ND
13.	**Xylose**	-	-	-	-	-	ND	ND	-	ND
14.	**Rhamnose**	-	ND	ND	AG	-	ND	ND	ND	ND
15.	**Inulin**	-	-	+	ND	-	ND	ND	ND	ND
16.	**Haemolysis**	+	-	+	+/-	ND	+	-	+	-
17.	**Coagulase**	+	-	+	NA	-	ND	ND	-	ND

Table-4 shows the incidence of various organisms isolated from mastitogenic cows

	Type of organisms	**No.of isolates**	**% of isolates**
1.	Streptococcus sp.	13	12.38
2.	Staphlococcus aureus	42	40.
3.	Staphylococcus epidermidis	11	10.48
4.	Bacillus sp.	4	3.81
5.	Corynebacterium sp.	2	1.90
6.	Pseudomonas sp.	2	1.90
7.	Escherichia coli	21	20
8.	Klebsiella spp.	5	4.76
9.	Proteus sp.	5	4.76

A total of 105 bacterial isolates were recovered from 96 milk samples obtained from 96 clinical and suspected obtained from 96 samples 42 samples (43.75%).Yielded pure cultures of which 9 (21.43%) were Gram positive and 33(78.57%) were Gram negative organisms and the remaining 54 (51.42%) samples yielded mixed cultures.

Of the 105 isolates 76(72.38%) were gram positive and remaining 29 (27.62%) were gram negative.The predominant bacterial isolates recovered were staphylococcus aureus(40%) and E.coli (20%) followed by staphylococcus epidermidis (10.48%) streptococcus species(12.38%) klebsiella species (4.76%) and pseudomonas (1.9%) respectively.

The frequency of isolation of different bacterial species from the suspected cases of mastitis cows was depicted in Table 2.

The high prevalence of staphylococcus species followed by E.coli in the present study is in agreement with the work of several earlier workers.Staphlococcus sp. are the most important mastitis causing organism globally including Orissa.Higher incidence of mastitogenic isolates may be due to poor hygienic practices as most of the bacterial infection originates from the cows environment and infect the udder via the teat canal.

Table-5 Depicted the sensitivity of bacterial isolates that isolated from mastitogenic cows to different antibiotics

Antibiotics	**Dose potency**	**Coliform spp.**	**Strepto spp.**	**Staphylo spp.**	**Proteus spp.**	**Corynebacterium spp.**
Tetracycline(T)	30 mcg	52.5%	75%	71.8%	70%	85%
Ampicillin(A)	10 mcg	3%	69%	79%	0%	86.5%
Neomycin(N)	30 mcg	12%	13.5%	24.6%	11.5%	12.5%
Erythromycin(E)	15 mcg	9%	81%	34%	49%	60.5%
Penicillin G(P)	10 units	3%	54.4%	72%	0%	80%
Ciprofloxacin(CF)	5 mcg	73%	70%	75%	25%	85%
Gentamicin(G)	10 mcg	85%	60%	65%	15%	75%
Doxycycline(DO)	30 mcg	12%	10%	13%	29%	11%

The relative frequency of different types of bacterial isolates status in Table 3.In the present study it is revealed that Coliform spp. Was highly sensitive to Gentamicin to 85% of isolates For Streptococcus spp. Tetracycline was

found to be 75% like was for Staphylococcus Ciprofloxacin (75%) for Proteus Tetracycline (70%) ,for Corynebacterium both the antibiotics Ampicillin (86.5%) and Ciprofloxacin (85%) respectively.All the mastitogenic isolates obtained were processed individually for antibiotic sensitivity test.

Table-6-MIC values of Methanolic extract of Green Tea samples (Diluted in Ethanol) in synergism (with tea samples and antibiotics)

Sr.No	Tea samples	MIC values in mg/ml
1	Green Tea + Chloramphenicol	10
2	Green Tea + Tetracycline	20
3	Green Tea + Levofloxacin	10
4	Green Tea +Gentamicin	10

In synergism ,the MIC of Green tea was enhanced with Chloramphenicol, Levofloxacin and Gentamicin

Table-7-MIC values of Methanolic extract of Black Tea samples (Diluted in Ethanol) in synergism (with tea samples and antibiotics)

Sr.No	Tea samples	MIC values in mg/ml
1	Black Tea + Chloamphenicol	10
2	Black Tea + Tetracycline	50
3	Black Tea + Levofloxacin	10
4	Black Tea + Gentamicin	10

In synergism, the MIC of Black tea was enhanced with Chloramphenicol, Levofloxacin and Gentamicin.

Table-8 Synergistic Antibacterial activity of crude methanol extract of green tea samples with chloramphenicol, tetracycline, levofloxacin and gentamicin antibiotics

Sr.No	Concentration mg/ml	Zone of inhibition (mm) of green tea with antibiotics			
		Chloramphenicol	Tetracycline	Levofloxacin	Gentamicin
1	5	-	16	17	14
2	10	-	18	17	16
3	15	-	17	13	14
4	20	-	19	18	15
5	25	14	16	18	16
6	30	13	17	16	16
7	35	17	18	17	17
8	40	17	16	19	17
9	45	15	16	19	18
10	50	18	19	19	19

The crude methanolic extract of green tea mixed with Tetracycline in same ratio (1:1) diluted in methanol showed good activity against staphylosoccus aureus at all concentrations. Mixing of extracts with chloramphenicol enhance the activities of tea samples. The crude methanolic extract of green tea when mixed with Chloramphenicol showed moderate results at the concentrations of 25-50 mg/ml but it gave poor results at other

concentrations. The synergistic activity of crude methanolic extract of green tea and Levofloxacin diluted in methanol showed best activity against staphylococcus aureus at all concentrations. The synergistic activity of crude methanolic extract of green tea and Gentamicin diluted in methanol showed best activity against staphylococcus aureus at all concentrations.The synergistic combination showed better result as compared to the green tea alone.

Table-9 Synergistic Antibacterial activity of crude methanol extract of black tea samples with chloramphenicol,tetracycline,levofloxacin and gentamicin antibiotics

Sr.No	Concentration mg/ml	Zone of inhibition (mm) of black tea with antibiotics			
		Chloramphenicol	Tetracycline	Levofloxacin	Gentamicin
1	5	12	15	18	15
2	10	-	18	18	16
3	15	-	16	12	12
4	20	-	17	17	15
5	25	13	18	19	14
6	30	12	15	15	15
7	35	15	18	18	16
8	40	15	16	19	15
9	45	14	17	19	16
10	50	17	18	18	18

The crude methanolic extract of black tea mixed with chloramphenicol in same ratio (1:1) diluted in ethanol showed good activity against *Staphylosoccus aureus* at all concentrations. Mixing of extracts with chloramphenicol enhance the activities of tea samples. The crude methanolic extract of black Tea when mixed with Chloramphenicol showed moderate results at the concentrations of 25-50 mg/ml but it gave poor results at other concentrations.The synergistic activity of crude methanolic extract of black tea and Levofloxacin diluted in methanol showed best activity against *Staphylococcus aureus* at all concentrations. The synergistic activity of crude methanolic extract of black tea and Gentamicin diluted in methanol showed best activity against *Staphylococcus aureus* at all concentrations.The synergistic combination showed better result as compared to the black tea alone.

Table-10 Antibacterial Activity of Methanol Extract of black Tea samples (Dilution in Methanol)

Sr No	Concentration mg/ml	Zone of inhibition(mm) Black Tea		
		Crude Methanol Extract	Crude Ethanol Extract	Synergistic antimicrobial activity of crude methanol extract (diluted in methanol)
1	5	-	-	15
2	10	-	-	17
3	15	-	-	15
4	20	-	-	16
5	25	-	-	15
6	30	14	-	13
7	35	11	10	13
8	40	12	-	15
9	45	11	-	20
10	50	19	-	15

It was concluded that methanol was the best solvent for extracting antimicrobial substances from Black tea samples. The synergistic activity of all methanolic extracts Black tea samples diluted in methanol showed good activity against *Staphylococcus spp*. at all concentrations.

Table-11 Antibacterial Activity of Methanol Extract of green Tea samples (Dilution in Methanol)

Sr.No	Concentration mg/ml	Zone of inhibition (mm) green Tea		
		Crude Methanol Extract	Crude Ethanol Extract	Synergistic antimicrobial activity of crude methanol extract (diluted in methanol)
1	5	-	-	14
2	10	-	-	19
3	15	-	-	16
4	20	-	-	17
5	25	-	-	16
6	30	16	-	14
7	35	12	10	11
8	40	14	-	17
9	45	13	-	15
10	50	19	-	17

It was concluded that methanol was the best solvent for extracting antimicrobial substances from Green tea samples. The synergistic activity of all methanolic extracts of Green tea samples diluted in methanol showed good activity against *Staphylococcus spp*.at all concentrations.

Table-12 Shows the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in mg/ml of different tea extracts

Pathogens	Green Tea						Black Tea					
	Methanol		Ethanol		Aqueous		Methanol		Ethanol		Aqueous	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Staphylococcus aureus	10	15	10	30	15	20	15	20	20	25	25	30
Staphylococcus epidermidis	10	15	10	30	10	15	15	20	20	25	25	30
Streptococcus spp	10	15	10	30	20	25	15	20	30	35	25	30
Escherichia coli	10	15	10	30	25	30	20	25	35	40	30	30
Pseudomonas spp.	10	15	10	30	45	50	20	30	30	40	25	30
Klebsiella spp.	No	No	No	No	No	No	No	No	No	No	No	No

The MIC for methanolic extracts of green tea was found to be 10 mg/ml for all types of test bacterial species except *Klebsiella spp.* The MIC for ethanolic extracts of green tea was found to be 10 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MIC for aqueous extracts of green tea was found to be 15 mg/ml for all types of test bacterial species except Klebsiella spp. The MBC for methanolic extracts of green tea was

found to be 15 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MBC for ethanolic extracts of green tea was found to be 30 mg/ml for all types of test bacterial species except *Klebsiella spp.* The MBC for aqueous extracts of green tea was found to be 15 mg/ml for all types of test bacterial species except *Klebsiella spp*.

The MIC for methanolic extracts of black tea was found to be 15 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MIC for ethanolic extracts of black tea was found to be 20 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MIC for aqueous extracts of black tea was found to be 25 mg/ml for all types of test bacterial species except *Klebsiella spp.* The MBC for methanolic extracts of black tea was found to be 20 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MBC for ethanolic extracts of black tea was found to be 25 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MBC for aqueous extracts of black tea was found to be 30 mg/ml for all types of test bacterial species except *Klebsiella spp*.

Table-13 Showed the data pertaining to zone of inhibition caused by different tea extracts

Types of Tea	Type of Extracts	Conc. mg/ml	E.coli spp.	Staph. spp.	Strepto. spp.	Klebsiella spp.	Pseudomonas spp.
Green Tea	**Aqueous**	25	5 mm	7 mm	6 mm	No zone	2 mm
		50	8 mm	9 mm	8 mm	No zone	3 mm
	Methanol	25	7 mm	8 mm	8 mm	No zone	3 mm
		50	9 mm	9 mm	9 mm	No zone	4 mm
	Ethanol	25	6 mm	6 mm	6 mm	No zone	2 mm
		50	8 mm	7 mm	7 mm	No zone	3 mm
Black Tea	**Aqueous**	25	4 mm	5 mm	4 mm	No zone	1 mm
		50	5 mm	6 mm	5 mm	No zone	2 mm
	Methanol	25	6 mm	6 mm	6 mm	No zone	1 mm
		50	8 mm	8 mm	8 mm	No zone	2 mm
	Ethanol	25	5 mm	5 mm	5 mm	No zone	1 mm
		50	6 mm	7 mm	6 mm	No zone	2 mm

Results obtained in the present study revealed that the tested two types of tea samples(green and black tea) posses potential antibacterial activity against *Staphylococcus aureus, Escherichia coli, Streptococcus spp*.,and *Klebsiella* (Table-13) .Each type of tea extract (aqueous, methanolic and ethanolic) of the two types of samples were tested at two different concentrations (25 and 50 mg/ml)to see their inhibitory effects against bovine mastitis isolated pathogens.

The most pronounced activity with inhibition zones of more than 8 mm was shown by methanolic extracts (inhibition zone 9 mm against S.aureus at concentration 50 mg/ml) and aqueous extract (inhibition zone 8 mm against E.coli at concentration 50 mg/ml) of green tea .The aqueous, methanolic and ethanolic extract of green and black tea both doesnot show

any zone of inhibition for Klebsiella spp.The zone of inhibition for *Pseudomonas* is 4 mm at a concentration of 50 mg/ml for methanolic exracts of green tea only .The black tea extracts show 1-2 mm zone of inhibition for Pseudomonas spp.When the concentration of extracts were decreased from 50-25 mg/ml, slight decrease in inhibition zones were observed.

DISCUSSION

Mastitis continues to be the single most expensive health related problem confronting the dairy industry worldwide. Based on several study by the researchers it has been recommended that dry cow therapy is the most effective therapy for preventing mastitis during dry period. Several workers pointed out that wide spread use of antibiotic resulted in development of resistant bacteria. Administration of plant derived antimicrobials have been reported not to induce resistance against both gram positive and gram negative bacteria after prolonged exposure (Domadia *et al* , 2007). Because plant derived molecules contain several different chemical groups in their structure, their antibacterial activity is not attributable to one specific mechanism. Milk is a complex medium in which lipophilic proteins such as albumin and other nutrients including fat and starch can potentially interact with the antimicrobial molecules there by reducing their bioavailability. The traditional ethno-veterinary medicinal practices are being followed by the rural folk through which a number of veterinary diseases are managed in the developing countries. The use of antibiotics and other chemical products are banned for animal healthcare in a number of countries because of human healthcare. The World Health Organization (WHO) states that 74% of the medicines derived from plant resources have a modern indication that correlates with their traditional, cultural (and sometimes ancient) uses (Ayyappa *et al*., 2009).

Here in we present data indicating the efficacy of tea (*Camellia sinensis*) leaves extract. Antibacterial activity of tea extracts has been reported by several workers (Toda *et al.*, 1991). From the above study a total of 105 bacterial isolates were recovered from 96 milk samples obtained from 96 clinical samples and it was found that from 96 samples

42 samples (43.75 %) Yielded pure cultures of which 9 (21.43 %) were Gram positive and 33 (78.57 %) were Gram negative organisms and the remaining 54 (51.42 %) samples yielded mixed cultures. The sources of milk collection was described in (Table -1).

Of the 105 isolates 76 (72.38 %) were gram positive and remaining 29 (27.62 %) were gram negative. 9Table-2) and (Table-3) shows the biochemical tests and sugar fermentation tests respectively for the test bacteria. The predominant bacterial isolates recovered were *Staphylococcus aureus* (40 %) and *Escherichia coli* (20 %) followed by *Staphylococcus epidermidis* (10.48 %) *Streptococcus* species (12.38 %) *Klebsiella* species (4.76 %) and *Pseudomonas* (1.9 %) respectively. The frequency of isolation of different bacterial species from the suspected cases of mastitis cows was depicted in (Table 4) and (Fig. No. 30).

The high prevalence of *Staphylococcus* species followed by *Escherichia coli* in the present study is in agreement with the work of several earlier workers. *Staphlococcus* spp. were the most important mastitis causing organism globally including Orissa. Higher incidence of mastitogenic isolates may be due to poor hygienic practices as most of the bacterial infection originates from the cows environment and infect the udder via the teat canal.

All the mastitogenic isolates obtained were processed individually for antibiotic sensitivity test. The relative frequency of different types of bacterial isolates status in (Table 5) and (Fig.No.29). In the present study it is revealed that *Coliform spp*. was highly sensitive to Gentamicin to (85 %) of isolates. For *Streptococcus spp*. Tetracycline was found to be (75 %) like was for *Staphylococcus spp*. Ciprofloxacin (75 %) for *Proteus*

spp. Tetracycline (70 %) ,for *Corynebacterium spp.* both the antibiotics Ampicillin (86.5 %) and Ciprofloxacin (85 %) respectively.

In light of these findings, we determined the Minimum inhibitory concentration (MIC) and (MBC) of various tea extracts against the mastitis pathogens. The antibacterial assay of aqueous and methanolic extracts was performed by (Bauer *et al*., 1996). Variations occur in zone diameter during the disk diffusion MIC methods due to temperature variations which influence the rate of inoculum growth as well as the diffusion rate of antibiotic. So incubation time variations was avoided. The incubation parameters like atmosphere, CO presence as well as humidity in the incubator and stacking of the plates were taken into consideration. An inoculum that will produce semi-confluent growth on the agar plate after overnight incubation was taken for calculation of MIC. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the tea extracts on *Streptococcus spp., Staphylococcus epidermidis, Staphylococcus aureus, and Escherichia coli* were determined.

Combinations of the extracts as shown in (Table 6 and 7) of *Camellia sinensis* leaves and different antibiotics was used to detect Synergy. The leaves extracts of the plant *Camellia sinensis* used in the present study showed prominent antibacterial activity against *Escherichia coli, Streptococcus spp., Staphylococcus spp* . and less activity against *Pseudomonas spp* and totally no effect on *Klebsiella spp*.

It was concluded from (Table-8 and 9) that methanol was the best solvent for extracting antimicrobial substances from Green or Black tea samples. The synergistic activity of methanolic extracts of both Green and Black tea samples diluted in methanol showed good activity against

Staphylococcus spp. at all concentrations. In synergism, both the Greenand Black tea was enhanced with Chloramphenicol, Levofloxacin and Gentamicin. The synergistic activity of crude methanolic extract of green tea and Levofloxacin diluted in methanol showed best activity against *Staphylococcus aureus* at all concentrations. The synergistic combination showed better result as compared to the green tea alone.

It was concluded from (Table-10 and 11) that methanol was the best solvent for extracting antimicrobial substances from Black tea samples. The synergistic activity of all concentrations of methanolic extracts of Black tea samples diluted in methanol showed good activity against *Staphylococcus* spp. at all concentrations. But this antimicrobial activity as compared to Green tea extract is less. In synergism, the Black tea was enhanced with Chloramphenicol, Levofloxacin and Gentamicin. The crude methanolic extract of black tea mixed with chloramphenicol in same ratio (1:1) diluted in ethanol showed good activity against *Staphylosoccus aureus* at all concentrations. Mixing of extracts with chloramphenicol enhance the activities of tea samples due to synergistic action. Usually, the combination of two agents exhibit significant potentiation (synergism) only if the test organism is resistant to at least one of the agents (Esimone *et al*., 2006). Perturbation of the cell membrane by tea extracts containing different (polyphenols) such as epicatechin, epigallocatechin results in a loss to free passage of materials in and out of cell leading to lysis of the cell, which eventually results in death of bacteria (Esimone *et al*., 2010).

The (Table-12) shows the MIC for methanolic extracts of green tea was found to be 10 mg/ml for all types of test bacterial species except *Klebsiella spp.* The MIC for ethanolic extracts of green tea was found to be 10 mg/ml for all types of test bacterial species except *Klebsiella spp*.

The MIC for aqueous extracts of green tea was found to be 15 mg/ml for all types of test bacterial species except *Klebsiella* spp. The MBC for methanolic extracts of green tea was found to be 15 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MBC for ethanolic extracts of green tea was found to be 30 mg/ml for all types of test bacterial species except *Klebsiella spp.* The MBC for aqueous extracts of green tea was found to be 15 mg/ml for all types of test bacterial species except *Klebsiella spp.*

The MIC for methanolic extracts of black tea was found to be 15 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MIC for ethanolic extracts of black tea was found to be 20 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MIC for aqueous extracts of black tea was found to be 25 mg/ml for all types of test bacterial species except *Klebsiella spp.* The MBC for methanolic extracts of black tea was found to be 20 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MBC for ethanolic extracts of black tea was found to be 25 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MBC for aqueous extracts of black tea was found to be 30 mg/ml for all types of test bacterial species except *Klebsiella spp*.

Results obtained in the present study revealed that the tested two types of tea samples (green and black tea) posses potential antibacterial activity against *Staphylococcus aureus, Escherichia coli, Streptococcus spp*., and *Klebsiella* (Table-13). Each type of tea extract (aqueous, methanolic and ethanolic) of the two types of samples were tested at two different concentrations (25 and 50 mg/ml) to see their inhibitory effects against bovine mastitis isolated pathogens.

The (Table-13) proved that crude methanolic extract of black Tea when mixed with tetracycline showed moderate results at the concentrations of 25-50 mg/ml but it gave poor results at other concentrations. The synergistic activity of crude methanolic extract of black tea and Levofloxacin diluted in methanol showed best activity against *Staphylococcus aureus* at all concentrations. The synergistic combination showed better result as compared to the black tea alone (Tahira *et al*., 2010).

The black tea extracts from (Table-13) show 1-2 mm zone of inhibition for *Pseudomonas spp*. When the concentration of extracts were decreased from 50-25 mg/ml slight decrease in inhibition zones were observed. The crude methanolic extract of black tea mixed with chloramphenicol in same ratio (1:1) diluted in methanol showed good activity against *Staphylosoccus aureus* at all concentrations. Mixing of extracts with chloramphenicol enhance the activities of tea samples (Tahira *et al*., 2010).

The most pronounced activity with inhibition zones of more than 8 mm was shown by methanolic extracts (inhibition zone 9 mm against *Staphylococcus aureus* at concentration 50 mg/ml) and aqueous extract (inhibition zone 8 mm against *Escherichia coli* at concentration 50 mg/ml) of green tea. The aqueous, methanolic and ethanolic extract of green and black tea both does not show any zone of inhibition for *Klebsiella* spp. The zone of inhibition for *Pseudomonas* is 4 mm at a concentration of 50 mg/ml for methanolic extracts of green tea only. The black tea extracts show 1-2 mm zone of inhibition for *Pseudomonas* spp. When the concentration of extract were decreased from 50-25 mg/ml, slight decrease in inhibition zones were observed. The most pronounced activity with inhibition zones of more than 8 mm was shown by

methanolic extracts (inhibition zone 9 mm against *Staphylococcus aureus* at concentration 50 mg/ml) and aqueous extract (inhibition zone 8 mm against *Escherichia coli* at concentration 50 mg/ml) of green tea.

The aqueous, methanolic and ethanolic extract of green and black tea both does not show any zone of inhibition for *Klebsiella spp.* The zone of inhibition for *Pseudomonas spp*. is 4 mm at a concentration of 50 mg/ml for methanolic extracts of green tea only.

From this study, tea extracts inhibited the growth of the pathogens tested and these include *Klebsiella spp. Pseudomonas spp.* as well as *Staphylococcus aureus, Streptococcus spp.* and *Escherichia coli* the most common causative agent of mastitis. These findings support the use of tea extracts in the treatment of mastitis caused by the above mentioned pathogens that are susceptible to the antibacterial activity of this plant. The extracts showed broad spectrum activity against both Gram-positive and Gram-negative bacterial strains (Tahira *et al*. 2010). The aqueous extract used in our investigations did not show activity against *Klebsiella spp*. of the test organisms suggesting that the active constituents of the tea (*Camellia sinensis*) leaves are not effective.

It is observed that methanol was the best solvent for extracting antimicrobial substances from tea samples based on the number of organisms inhibited and the diameter of inhibitory zones produced. It could also be seen that different extracts were different in their antimicrobial effectiveness depending on the extractive solvent used. These results favour that bioactive components of any medicinal plant may differ in their solubility depending on the extractive solvents used. The water extract was least bactericidal as compared to other solvent extracts and showed medium activity against *Staphylococcus spp*.

Thus the use of tea extracts in the treatment of mastitis associated with the infection of these pathogens is validated and scientifically supported by the results obtained in this work. The synergy against, *Staphylococcus spp., Streptococcus spp. Escherichia coli, Pseudomonas spp.* is noteworthy as these bacteria were resistant to Penicillin G, Chloramphenicol, Amoxycillin, Oxytetracycline, Ciprofloxacin and Erythromycin with MIC values much higher than their predicted breakpoints. Although the level of antibiotic potentiation was low as not to lead to a restoration of susceptibility (lowering the MIC values to below the breakpoint values) the results seem promising considering that crude extracts were used. The potentiation is likely to have been much more pronounced if pure compounds were used instead of crude extracts in combinations against the test bacteria. The MIC method also revealed the ability of the extract to improve the bactericidal effects of the antibiotics on both Gram negative and Gram positive bacteria (Tahira *et al*., 2010). Therefore, tea extracts can be used for further studies to be an effective antimicrobial as a direct intra mammary infusion under field conditions, so it should maintain its antimicrobial activity over an extended period. However, the MIC and MBC experiments determined the antimicrobial activity of tea extracts in milk is only up to 24 hr. Moreover tea can get degraded or inactivated in milk over a period of time. Therefore the persistence of antimicrobial activity of tea extracts on *Staphylococcus aureus* in milk for few days to confirm if tea extracts could maintain antimicrobial activity over this period. *Staphylococcus aureus* was chosen for this experiment because *Staphylococcus aureus* (along with *Escherichia coli* and *Streptococcus* spp.) required the highest MIC and MBC of tea extracts compared with the other pathogens. Moreover, *Staphylococcus aureus* is more commonly associated with contagious mastitis than the other species. Moreover, the invasion of

pathogens into the mammary gland could occur multiple times after the intramammary infusion of antimicrobials. Therefore, to investigate whether tea extracts would maintain its effectiveness over a period of time in a milk environment, *Staphylococcus aureus* was used in the experiment (Ananda Bhaskaran *et al*., 2009). In fact, given the antimicrobial activity documented here, tea extracts may also be useful in controlling mastitis during lactation (Ananda Bhaskaran *et al*., 2009). However, future experiments are needed to determine the pharmacokinetics of tea extracts in bovine mammary gland studies, and to ascertain the in vivo efficacy of this molecule for treating bovine mastitis as an intramammary treatment, in addition to its potential effect on the mammary gland tissue (Ananda Bhaskaran *et al*. 2009).

(Wynn *et al*., 2001) describes today's traditional medicine, as undoubtedly the oldest form of medicine and probably had evolved simultaneously with the evolution of human beings. EVM has been a mainstay of developing countries that lack access to conventional medicines for veterinary health care, often the only unaffordable means to poor farmers. The Ethno veterinary medicine (EVM) practices could be an effective approach for tackling problems like mastitis in bovine. With the traditional knowledge in the background potential plants can be prospected to reach the active fraction or molecules, which can be further formulated. Besides, the tea extracts itself could be utilized, by farmers as intra mammary infusion. Further studies are necessary to elucidate the specific phytoactive compounds in the leaf extract of tea.

PLATE NO. 11

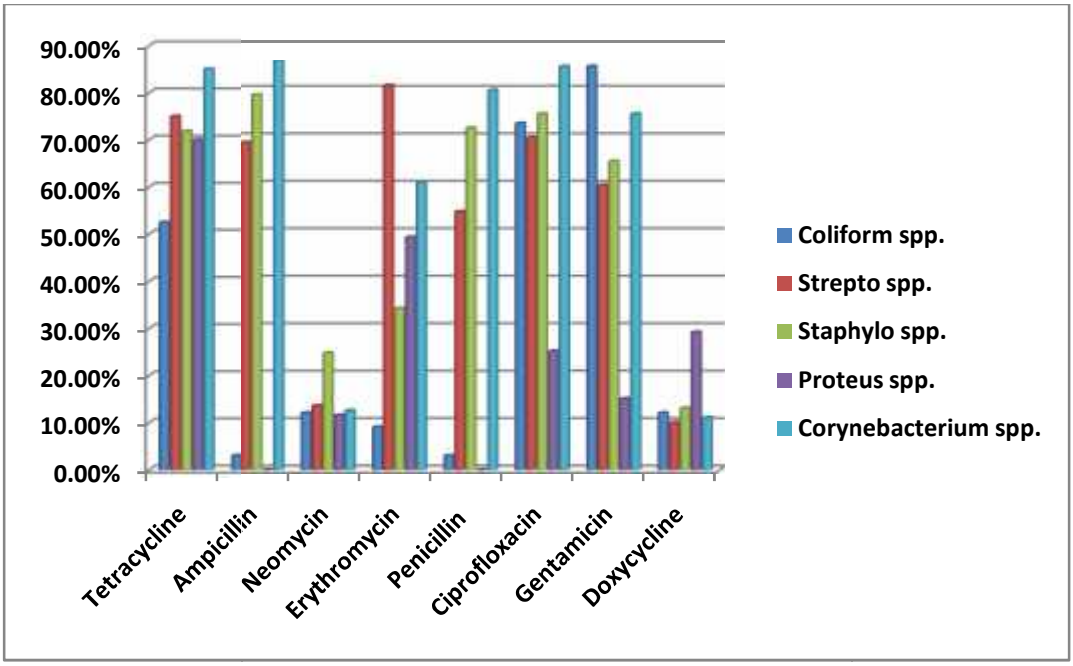

Fig no 29: Depicted the sensitivity of bacterial isolates that isolated from mastitogenic cows to different antibiotics

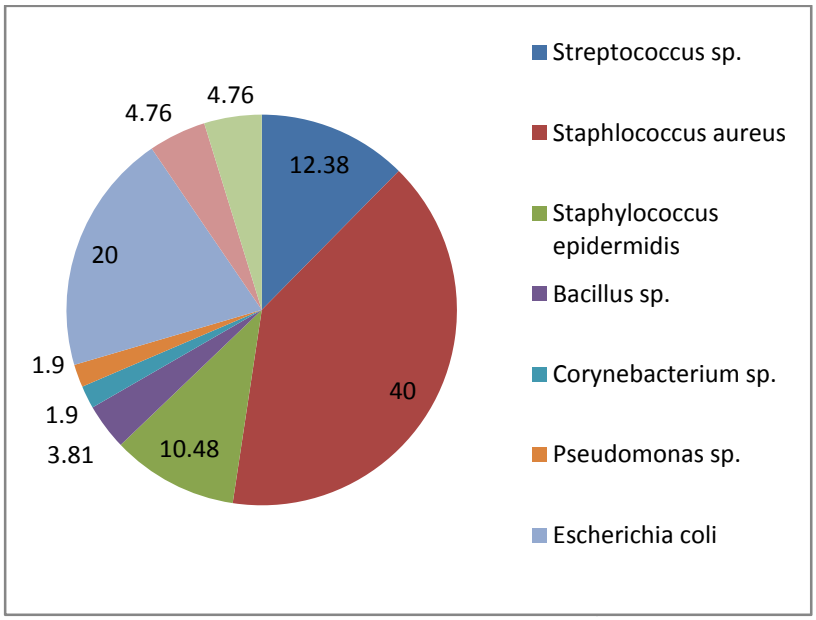

Fig no 30: Incidence of various organisms (in %) isolated from mastitogenic cows

VI. SUMMARY

1. In the present study it was found that out of the 105 isolates 76 (72.38 %) were gram positive and remaining 29 (27.62 %) were gram negative. The predominant bacterial isolates recovered were *Staphylococcus aureus* (40 %) and *Escherichia coli* (20 %) followed by *Staphylococcus epidermidis* (10.48 %) *Streptococcus* species (12.38 %) *Klebsiella* species (4.76 %) and *Pseudomonas* (1.9 %) respectively.

2. The high prevalence of *Staphylococcus* species followed by *Escherichia coli* in the present study is in agreement with the work of several earlier workers. *Staphlococcus* spp. were the most important mastitis causing organism globally including Orissa. Higher incidence of mastitogenic isolates may be due to poor hygienic practices as most of the bacterial infection originates from the cows environment and infect the udder via the teat canal.

3. In the present study it is revealed that *Coliform spp*. was highly sensitive to Gentamicin to (85 %) of isolates. For *Streptococcus spp*. Tetracycline was found to be (75 %) like was for *Staphylococcus spp*. Ciprofloxacin (75%) for *Proteus spp*. Tetracycline (70%), for *Corynebacterium spp.* both the antibiotics Ampicillin (86.5 %) and Ciprofloxacin (85 %) respectively.

4. The MIC for methanolic extracts of green tea was found to be 10 mg/ml for all types of test bacterial species except *Klebsiella spp.* The MIC for ethanolic extracts of green tea was found to be 10 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MIC for aqueous extracts of green tea was found to be 15 mg/ml for all types of test bacterial species except *Klebsiella* spp.

5. The MBC for methanolic extracts of green tea was found to be 15 mg/ml for all types of test bacterial species except *Klebsiella spp*. The MBC for ethanolic extracts of green tea was found to be 30 mg/ml for all types of test bacterial species except *Klebsiella spp.* The MBC for aqueous extracts of green tea was found to be 15 mg/ml for all types of test bacterial species except *Klebsiella spp*.

6. The synergistic activity was found at all concentrations of methanolic extracts of Green and Black tea samples diluted in methanol against *Staphylococcus spp*. In synergism, the Green and Black tea was enhanced with antibiotics like Chloramphenicol, Levofloxacin and Gentamicin.

7. The aqueous, methanolic and ethanolic extract of green and black tea both does not show any zone of inhibition for *Klebsiella spp.* The zone of inhibition for *Pseudomonas spp*. is 4 mm at a concentration of 50 mg/ml for methanolic exracts of green tea only.

8. From this study, tea extracts inhibited the growth of the pathogens tested and these include *Klebsiella spp. Pseudomonas spp.* as well as *Staphylococcus aureus, Streptococcus spp*. and *Escherichia coli* the most common causative agent of mastitis. These findings support the use of tea extracts in the treatment of mastitis caused by the above mentioned pathogens that are susceptible to the antibacterial activity of this plant. The extracts showed broad spectrum activity against both Gram-positive and Gram-negative bacterial strains. The aqueous extract used in our investigations did not show activity against *Klebsiella spp*. of the test organisms suggesting that the active constituents of the tea (*Camellia sinensis*) leaves are not effective.

9. *Staphylococcus aureus* was chosen for this experiment because *Staphylococcus aureus* (along with *Escherichia coli* and *Streptococcus* spp.) required the highest MIC and MBC of tea extracts compared with the other pathogens.

10. In order to establish and explore methanolic extracts as an antimastitic agent a vivid study is required.

VII. CONCLUSION

From the above study it is concluded that the incidence of clinical and subclinical mastitis in cross bred cows is a common problem for costal and remote areas of Orissa in summer season. The pathogenic *Staphylococcus* spp. and *Streptococcus* spp. are the principal pathogens causing mastitis. Tea extracts have great potential as antimicrobial compounds. The synergistic effect from the association of antibiotic with tea extracts against resistant bacteria leads to new choices for the treatment of mastitis. This study has shown that crude extracts of the leaves of *Camellia sinensis* exhibits potentials of synergy in combination with some antibiotics against pathogenic bacteria often presenting with problems of drug resistance. This synergistic attributes of crude extracts of *Camellia sinensis* leaves and antibiotics demonstrates that this plant possess antibiotic resistance modifying compounds. In conclusion, tea extracts did not impair the antimicrobial properties of antibiotics; rather, it enhanced its activity in an additive manner which could be beneficial in the treatment of staphylococcal infections.

In the present study it was revealed that methanol was the best solvent for extracting antimicrobial substances from Green and Black tea samples. The synergistic activity of all methanolic extracts of Green and Black tea samples diluted in methanol showed good activity against most of the test bacteria except few at all concentrations. In synergism, the Black tea activity was enhanced with Chloramphenicol, Levofloxacin and Gentamicin. It is suggested that both the types of tea samples used in the study could be explored for possible antimicrobial agents for various mastitis causing bacteria. In order to establish the above finding a more elaborative study in mastitic isolates is needed from different geographical areas.

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PLATE NO. 1

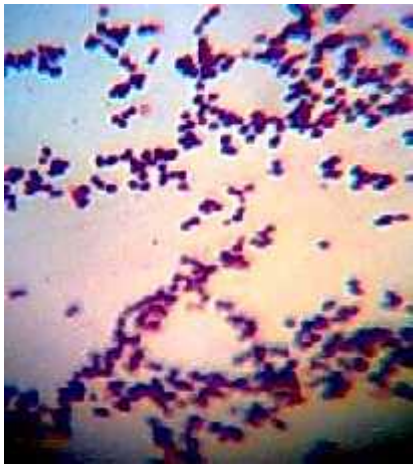


Fig.no.1.*Staphylococcus spp*. in Gram`s Staining.

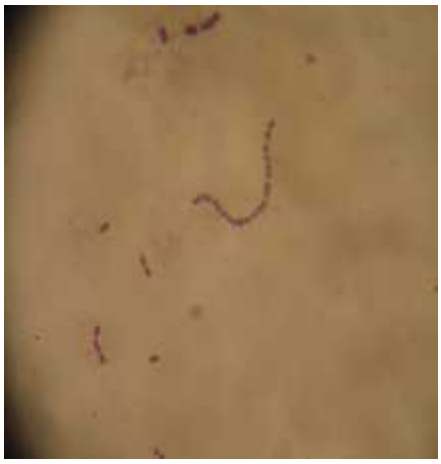


Fig no.2. *Streptococcus spp.* in Gram`s Staining.

Fig no.3. Gm –ve *Bacilli* from milk sample in Gram`s stain

PLATE NO. 2

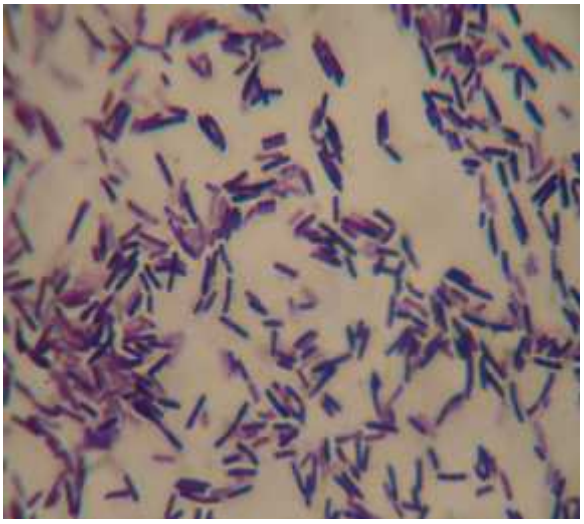


Fig no 4. Gram Positive Bacilli in Gram Staining

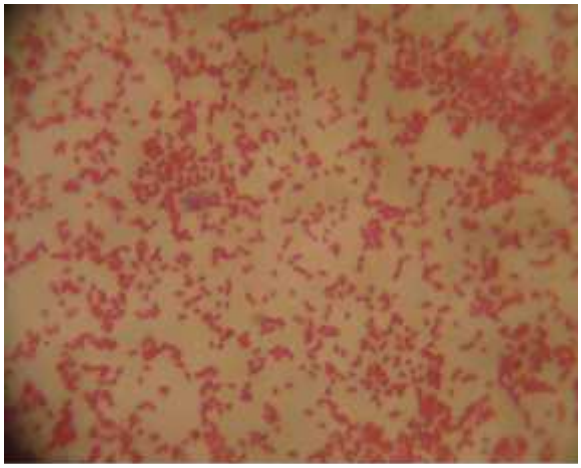


Fig no 5. Gram Negative Coccobacilli in Gram's Stain

Fig no 6.*Corynebacterium Spp.* from milk sample in Gram`s stain

PLATE NO. 3

Fig no 7. *Klebesiella s*pecies in Gram`s Stain Isolated from milk
.

Fig no 8.*Staphylococcus* growth in Manitol Salt Agar
.

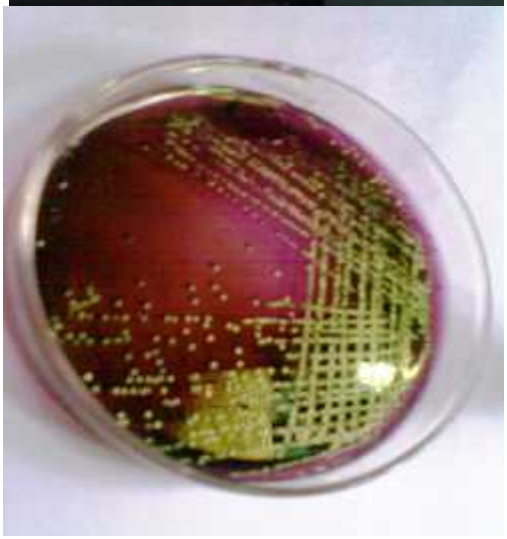


Fig no9. *E.coli* grown in EMB Agar Isolated from milk sample having metallic sheen.

PLATE NO. 4

Fig no 10. MLA , Lactose fermentor Pink Colony. E.coli.

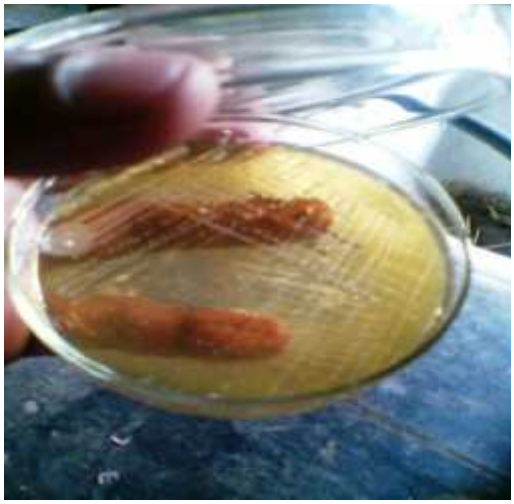


Fig no.11.*Pseudomonas spp*. in Pseudomonas Isolation Agar
.

Fig no 12. Taking Single Colony of E.coli for minimum inhibitory concentration (MIC) Calculation

PLATE NO. 5

Fig no 13. Sugar tests for Staphylococcus aureus..

Fig no.14. Sugar tests for E.coli .

PLATE NO. 6

Fig no 15
(a)VP test : positive,
(b)Indole test : positive

Fig no 16. MR test:
(a) positive (b) negative

Fig no 17. Citrate Test
A- Green Color (Negative)
B- Blue Color (Positive)

PLATE NO. 7

Fig no 18. H_2S test:
(a) & (b) positive for Proteus.
(c) negative for others
.

Fig no 19. Oxidase test for *Pseudomonas aurugenosa*

Fig no 20. Catalase test

PLATE NO. 8

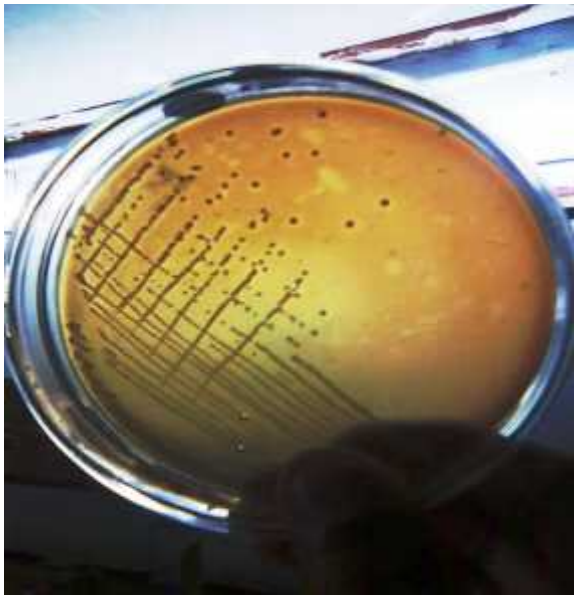


Fig no 21. ***Staphylococcus aureus in Blood Agar showing zone of heamolysis***

Fig no 22. Streaking of Milk Sample for Isolation

Fig no 23. Antibiotic Sensitivity Test of Milk Sample

PLATE NO. 9

Fig no 24: Minimum inhibitory construction (MIC) calculation of green tea extract for *E.coli*
AQ1: Aqueous green tea extract at concentration 100mg /ml.
AQ2 : Aqueous green tea extract at concentration 200mg/ ml.
Ex : Enrofloxacin (sensitive).
Ce : Cefotaxime (Resistant)

Fig no 25: Minimum inhibitory construction (MIC) calculation of black tea extract for *E.coli*
AQ1: Aqueous black tea extract at concentration 10mg /ml.
AQ2 : Aqueous black tea extract at concentration 20mg/ ml.
Ex : Enrofloxacin (sensitive).
Ce : Cefotaxime (Resistant)

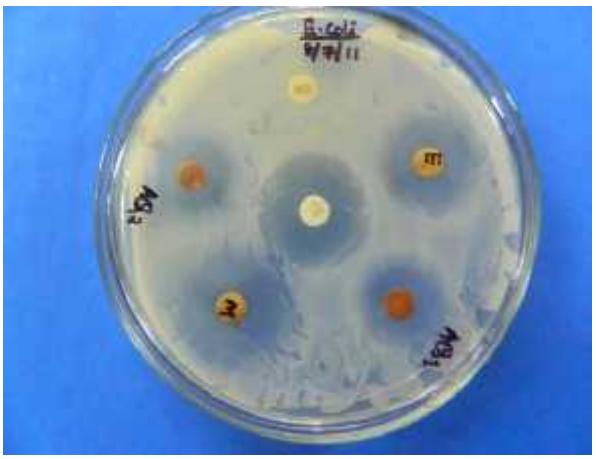


Fig no 26: Minimum inhibitory construction (MIC) calculation of black tea extract for *E.coli*
AQ3: Aqueous black tea extract at concentration 30mg /ml.
AQ4 : Aqueous black tea extract at concentration 40mg/ ml.
Ex : Enrofloxacin (sensitive).
Ce : Cefotaxime (Resistant)

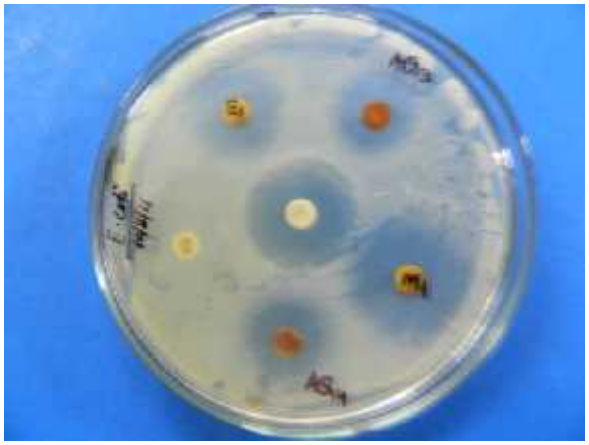


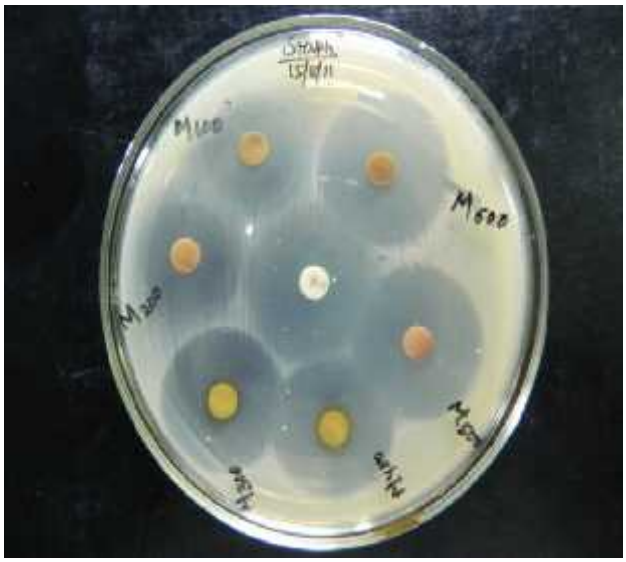
PLATE NO. 10

Fig no 27: Minimum inhibitory construction (MIC) calculation of green tea extract for *Staphylococcus spp.*

A100: Aqueous extract at concentration 10 mg /ml.
A200 : Aqueous extract at concentration 20 mg/ ml.
E100 : Ethanolic extract at concentration 10 mg/ml.
E200 : Ethanolic extract at concentration 20 mg/ml.
M100 : Methanolic extract at concentration 10 mg/ml.
M200 : Methanolic extract at concentration 20 mg/ml.

Fig no 28: Minimum inhibitory construction (MIC) calculation of green tea Methanolic extract for *Staphylococcus spp.*

M100: Methanolic extract at concentration 10 mg /ml.
M200 : Methanolic extract at concentration 20 mg/ ml.
M300 : Methanolic extract at concentration 30 mg/ml.
M400 : Methanolic extract at concentration 40 mg/ml.
M500 : Methanolic extract at concentration 50 mg/ml.
M600 : Methanolic extract at concentration 60 mg/ml.



APPENDIX

1. BRAIN HEART INFUSION AGAR

Calf brain infusion (solids)	200.00 gm
Beef heart infusion (solids)	250.00 gm
Protease peptone	10.00 gm
Sodium chloride	5.00 gm
Dextrose	20.00 gm
Disodium phodphate	2.50 gm
Distilled water	1000 ml

Final pH was adjusted to 7.4 and autoclaved at 15 lb pressure for 15 minutes.

2. MULLER HINTON AGAR

Beef infusion from 300 gm meat

Casein hydrolysate 17.5 gm

Starch 1.5 gm

Agar 17 gm

Distilled water 1000 ml

The ingredients were dissolved by boiling. pH adjusted to 7.4 and then autoclaved at 15 lb pressure for 15 minutes.

3. BLOOD ARAR MEDIA (BA)

i) Nutrient Agar (NA)

meat extract 3 gm

peptone 10 gm

sodium chloride 5 gm

agar powder 20 gm

distilled water 1000 ml

The ingredients were dissolved by boiling 20 minutes. Then the pH was adjusted to 7.4 and then autoclaved at 15 lb pressure for 30 minutes.

ii) blood agar (BA)

The NA was cooled to 50^0 C and defibrinated sheep blood @ 7.5 % was added to it and mixed thoroughly. The media was distributed in 5 ml quantity in test tube for BA slants and 20 ml in petri dishes (10 cm diameter) for a BA plate. Sterility was checked by overnight incubation and the plate and slants were stored at 4^0 C for use in need.

4. CRYSTAL VIOLET BLOOD AGAR

Selective media for Streptococci

0.02 per cent aqueous solution of crystal violet 1 ml

Blood agar 100 ml

5. MA CONKEYS LACTOSE AGAR (MLA)

Peptone 2gm

Sodium taurocholate (bile salt) 0.5 gm

Sodium chloride (Nacl) 0.5 gm

Lactose 1.0 gm

Agar power 2.0 gm

Netural res (1% soln) 0.3 ml

Crystal violet (0.1% soln) 0.1 ml

Distilled water added up to 100 ml

The first 5 ingredients were added to distilled water and dissolved by boiling. Then neutral red was added. pH was adjusted to 7.4 and then sterilized by autoclaved at 15 minutes.

6. MANITOL SALT AGAR

Yeast extract 1g

Peptone 10 g

D-Mannitol 10 g

Sodium chloride 75 g

Phenol red 0.025 g

Agar 20 g

Distilled water 1000 ml

Final pH (at 25°C) 7.4+/- 0.2 and autoclaved at 15 lb pressure for 15 minutes.

7. PEPTONE WATER

Peptone 1gm

Sodium chloride 0.5gm

Distilled water 100ml

The ingredients were dissolved by boiling and cooled. The pH was adjusted to 7.2-7.4. Sterilized by autoclaved at 15 lb pressure for 15 minutes.

8. MOTILITY MEDIUM

Peptone 10gm

Meat extract 3gm

Sodium chloride 5gm

Agar 4gm

Distilled water 100ml

The ingredients were dissolved by boiling and cooled. pH was adjusted to 7.4. Then 10 ml amounts were dispended to the test tube and autoclaved at 121°C for 15 minutes and left to set in the vertical position.

9. NUTRIENT BROTH

Peptone 1gm

Sodium chloride 0.5gm

Potassium nitrate 0.5gm

Distilled water 100ml

The ingredients were dissolved by boiling and cooled. pH was adjusted to 7.4 and autoclaved at 15 lb pressure for 15 minutes.

10. NUTRIENT GELATINE MEDIUM

Peptone 10 gm

Sodium chloride 5 gm

Beef extract 3 gm

Nutrient gelatin 120 gm

Distilled water 100 ml

The ingredients were dissolved by warming and sterilized in flowing steam (60°C) for 10 minutes for 3 consecutive days and stored at 4°C.

11. TRIPLE SUGAR AGAR

Peptone 10 gm

Tryptone 10 gm

Lactose 10 gm

Saccharose 10 gm

Yeast extract 3 gm

Dextrose 1 gm

Ferrous sulphate 0.2 gm

Sodium chloride 5 gm

Sodium trisulphate 0.3 gm

Phenol red 0.024 gm

Agar 12.0 gm

Distilled water 1000 ml

12. SIMMONS CITRATE AGAR

Sodium chloride 5.0 gm

Magnesium sulphate 0.2 gm

Ammonium dihydrogen phosphate 1.0 gm

Potassium dihydrogen phosphate 1.0 gm

Sodium citrate 5.0 gm

Agar 20.0 gm

Distilled water 1000 ml

Bromothymol blue (0.2%) 40 ml

Ingredients were dissolved in distilled water and pH was adjusted to 6.8 and autoclaved at 121°C for 15 minutes. Then dispenced in tubes for preparation of slants.

13. KOVACS REAGENT

P-dimethyl-aminobenzaldehyde 10 gm

Amyl alcohol 150 ml

Concentrated hydrochloride 50 ml

14. NITRATE TEST REAGENT

Solution A

Sulphanilic acid 1 gm

5N-Acetic acid 125 ml

Solution B

A-N-Acetic acid 1 gm

5N-Acetic acid 200 ml

Equal volume of solution"A" & "B" were mixed to make nitrate test reagent. 5N-Acetic acid was prepared by adding 28.75 ml of gallic acid in 71.25 ml of distilled water.

15. ANDRADES INDICATOR

It was made by adding 1N sodium hydroxide to 0.5 percent solution of Acid fuchsin until the colour just became yellow.

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