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In vitro Impact of Indian Propolis (Bulandshahr, Uttar Pradesh) and Antibiotics on *Staphylococcus spp*. Isolated from Milk of Cows with Mastitis

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A B S T R A C T

Mastitis is one of the major factors obstructing the progress of the dairy industry. The use of antibiotics has been related with antibiotic-resistant pathogens. Consequently, several studies have been done in order to find natural alternatives. One of these alternatives is propolis which has been identified as an appropriate option according to recent studies of antibacterial, antiviral, antifungal, and antiprotozoal activity. The aim of this study was to test In Vitro the antimicrobial activities of some commercial antibiotics and methanol extract of Indian propolis (MEIP) against Staphylococcus spp. Isolated from mastitis-infected cow milk from the Gowshala (dairy farm), Department of Animal Husbandry and Dairying, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India. The effectiveness of antibiotics and MEIP in inhibiting growth of Staphylococcus spp. was Ciprofioxacin 5µ, Norfloxacin 10µ, Erythromycin 15µ, Doxyciclin 30µ, Lincomycin 2µ, Offloxacin 5µ and Cefalexin 30µ are susceptible, while Bactrin 25µ, Ceftioxone 30µ, Gentamycin 10µ, Clarithromycin 15µ and Azithromycin 15µ besides MEIP are intermediate and Cefradine 30µ, Cloxacillin 30µ and Amoxycillin 10µ are resistant.

Introduction

Milk production is an important agricultural sector in India which plays a significant role in the Indian economy, and successful endeavors of crossing local low-milk-yielding cattle with lactating cows have been producing animals of high milk potential, the physiological stress and strain of heavy milk production, deep caudo-ventral placement of udder, large size and carriage of large quantity of milk by the udder makes them more sensitive and prone to injury and subsequent udder infection and inflammation resulting into mastitis (Joshi 2006). Subclinical mastitis is a flock issue because it includes a reservoir of infections which could be transmitted to other animals of a herd. While clinical mastitis is an individual problem and it is characterized by changes in the udder and the milk drawn from it. An average decrease in milk yield was estimated as 50% due to the clinical mastitis and 17.5%

due to the subclinical mastitis (Joshi 2006). Mastitis is a main factor that is obstructing the progress required in the dairy industry, which is one of the destructive elements of the economy (Erskine *et al*., 2003; McDougall *et al*., 2009). Anti-mastitis is usually treated during the period of lactation or none-lactating by direct injection into the udders.

Nevertheless, the use of antibiotics in animal husbandry has been related with selection of antibiotic-resistant pathogens and the presence of antibiotic residues in the food chain (Van Eenennaam *et al*., 1993; Nickerson 2009). Consequently, many countries prohibit the use of antibiotics in raising livestock and restrict the importation of products derived from antibiotic-treated animals (Oeztuerk; Sagmanligil 2009). As a consequence, several studies have been done in order to discover alternative feed additives, which are natural and accepted by consumers such as natural antimicrobial products. Among these, propolis has attracted much attention in recent years because of its antibacterial, antiviral, antifungal, and antiprotozoal activity and its antibacterial activity was mainly ascribed to flavonoids or to a synergism between some components.

The structure of propolis is a phrase of corresponding concentrations of its component depends on the variety of the honeybee Apis Mellifera as well as the phytogeographic characteristics around the beehive (Marcucci 1995). Propolis structure is extremely complex: its main constituents are beeswax, resin and volatiles, but also pollen, amino acids, flavonoids (major components: rutin, quecertin, galangin) and caffeic acid phenetyl ester were identified, among others (Kumazawa *et al*., 2004). In other words, propolis is a complex mixture of bee released compounds and resin of plant. The proportion of the different substances in the propolis depend on the place and time of collection. In broad terms, raw propolis is consisting of approximately (50% resins, 30% waxes, 10% essential oils, 5% pollen and 5% of various organic compounds) (Salation *et al*., 2005). The chemical structure of propolis is complex; flavonoid and (hydroxyl) cinnamic acid derivatives are considered to be the essential biologically active constituents in propolis extract. It also includes many different chemical components according to geographical regions, climate and bee strain (Bankova 2005). Due to the complex structure of propolis it cannot be used as raw material but it can be used properly after extraction with several types of solvents which take away the inert material and preserve the desired compounds. The widespread solvents that are utilizing for extracting propolis as follows: methanol, ethanol, water, chloroform, dichloromethane, acetone and ether. The antimicrobial activities of some commercial antibiotics and methanol extract of Indian propolis (MEIP) have been tested In Vitro against *Staphylococcus* spp. isolated from mastitis-infected cow milk from the Gowshala (dairy farm), Department of Animal Husbandry and Dairying, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India.

Materials and Methods

The milking herd of dairy cows of the Gowshala (dairy farm), which is including 90 lactating cows (Indigenes cross-bred and lactating cows), Department of Animal Husbandry and Dairying, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India have been selected to investigate for mastitis during milking period.

Milk sample

The milk samples were taken from the cows which are infected with mastitis from the flock of the Gowshala (dairy farm), for isolating *Staphylococcus* spp., which is considered the

essential reason behind the infection of mastitis.

Bacterial isolates and inoculum preparation

To identify and isolate the bacteria, 0.1 mL of milk sample were spread onto 8% blood agar plates and then incubated overnight at 37°C. The microbial strains were presumptively identified on the basis of morphology, hemolysis pattern and Gram staining of the colonies, and then colonies of each microbial strain were streaked on blood agar to obtain a pure culture. After identification, colonies of *Staphylococcus* spp. were stored at -20°C in Brain Heart Infusion agar with glycerol.

Origin propolis

Propolis sample was collected in December 2016 to March 2017 from Bulandshahr district in the Meerut region in the North India state of Uttar Pradesh located between Ganga and Yamuna rivers, which is situated 77.0° and 78.0° east longitudes and between 28.4° south and 28.0° north latitudes. The district is 237.44 meters above sea level.

Propolis Extracts

Propolis has a complicated composition and cannot be utilized directly in the raw form. The usual process is the use of a solvent (National Committee for Clinical Laboratory Standards, 2003), which must strip the inert material and maintain the required compounds. So the major solvents used for extraction of bioactive contents are ethanol, methanol and water. Crude propolis contains plant resins, wax and insoluble substance. There are many methods of extracting the biological components of propolis. The sample of propolis were kept at -30˚C for 24 hours than crush to fine powder and then extracted in 97% methanol with shaking twice in day at room temperature for 14 days, after that the methanol solution was filtered through a filter paper (Whatman No. 41)

California Mastitis Test (CMT)

The California mastitis test uses to determine somatic cell counts (SCC) which measure the health of cow's udder. The DNA reacting with the test reagent which disrupts the cell membrane of somatic cells in the milk sample. The test has been conducted on the lactating herd of the Gowshala (dairy farm) Banaras Hindu University to identify the cows with mastitis.

Antimicrobial Susceptibility

The examinations antimicrobial activity of Methanol Extract of Indian Propolis (MEIP) were proceeded by the disc diffusion method based on the guidelines of the National Committee for Clinical Laboratory Standards (2003). Several commercial antibiotics were also tested under the same condition, specifically: Ciprofioxacin 5µ, Norfloxacin 10µ, Bactrin 25µ, Ceftioxone 30µ, Gentamycin 10µ, Cefradine 30µ, Cloxacillin 30µ, Erythromycin 15µ, Doxyciclin 30µ, Lincomycin 2µ, Offloxacin 5µ, Cefalexin 30µ, Clarithromycin 15µ, Azithromycin 15µ and Amoxycillin 10µ.

Determination of Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) was identified as the lowest concentration of the Indian propolis extract which prevented the growth of the examined microorganisms. All groups were noticed visually and MIC values were registered as had no visible turbidity.

Data analysis

The data represent observations from at least duplicate samples obtained from two experiments carried out independently.

Results and Discussion

Rate of occurrence of Mastitis based on (CMT) and Microbiological examination

The herd of dairy cows of the Gowshala (dairy farm), which is including 90 lactating cows have been investigated, and the results of California Mastitis Test (CMT) showed that 53 subclinical and 2 clinical mastitis.

Of the 53 milk cow samples with subclinical mastitis, in a scale of scores from 1 to 5, 55.4% showed scores 2 and 3, while the microbiological analysis appeared only 8.3%. Furthermore, the other samples 44.6% showed scores 4 and 5, and only 6.3% conformed by microbiological analysis.

Effectiveness of antibiotics and MEIP in inhibiting growth of *Staphylococcus* spp.

The effectiveness of antibiotics and Methanol Extract of Indian Propolis (MEIP) in inhibiting growth of *Staphylococcus* spp. presented in figure 1 which clarified that Ciprofioxacin 5µ, Norfloxacin 10µ, Erythromycin 15µ, Doxyciclin 30µ, Lincomycin 2µ, Offloxacin 5µ and Cefalexin 30µ have higher effectiveness in inhibiting of *Staphylococcus* spp. While Bactrin 25µ, Ceftioxone 30µ, Gentamycin 10µ, Clarithromycin 15µ and Azithromycin 15µ besides MEIP are identified moderate effectiveness in inhibiting of *Staphylococcus* spp. Whereas Cefradine 30µ, Cloxacillin 30µ and Amoxycillin 10µ did not show any effectiveness in inhibiting of *Staphylococcus* spp.

In light of table 1 and figure 2 antibiotics and Methanol Extract of Indian Propolis (MEIP) in this study have been categorized into three groups susceptible, intermediate, and resistant antibiotic accordingly to inhibit the growth of *Staphylococcus* spp. The first group is including susceptible antibiotics, as the diameter of inhibitions growth of *Staphylococcus* spp. by Doxyciclin 30µ (30mm), Cefalexin 30µ (27mm), Norfloxacin 10µ (26mm), Ciprofioxacin 5µ (25mm), Erythromycin 15µ (23mm), Offloxacin 5µ (23mm) and Lincomycin 2µ (22mm). Whereas the second group is including intermediate antibiotics, as the diameter of inhibitions growth of *Staphylococcus* spp. by Bactrin 25µ (15mm), Ceftioxone 30µ (12mm), Gentamycin 10µ (13mm), Clarithromycin 15µ (14mm) and Azithromycin 15µ (13mm), as well as MEIP has been identified as intermediate which its diameter of inhibitions growth is measured as (11mm). However, the third group is represented in resistant antibiotics, which is comprising: Cefradine 30µ, Cloxacillin 30µ and Amoxycillin 10µ, considering that they did not show any effect.

Pinto, *et al*., (2001) define the impact of green propolis extracts on bacteria isolated from milk of cows with mastitis that the inhibition zones (8.7-11 mm).

The susceptibility of Methanol Extract of Indian Propolis (MEIP) in inhibiting growth of *Staphylococcus* spp. has been estimated at between 9mm and 11mm, as shown in figure 3.

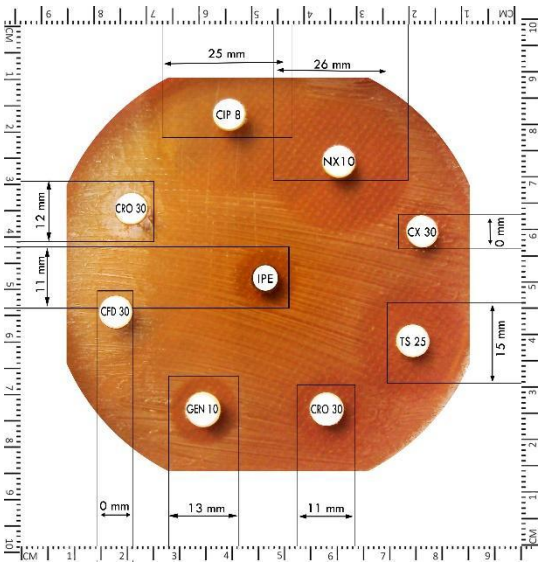
Santos Neto *et al*., (2009) found that for both the ethanolic and aqueous extracts of the green propolis, MIC was determined for the isolate of *Staphylococcus* spp. that showed the largest diameters of the inhibition zones.

On the other hand, the consequence of the slight solubility of propolis components (phenolics, flavonoids, among others), Pinto, *et al*., (2001) found that aqueous extract of propolis did not appear any in vitro antimicrobial effect against strains of *Staphylococcus* aureus isolated from milk of cows suffering from mastitis.

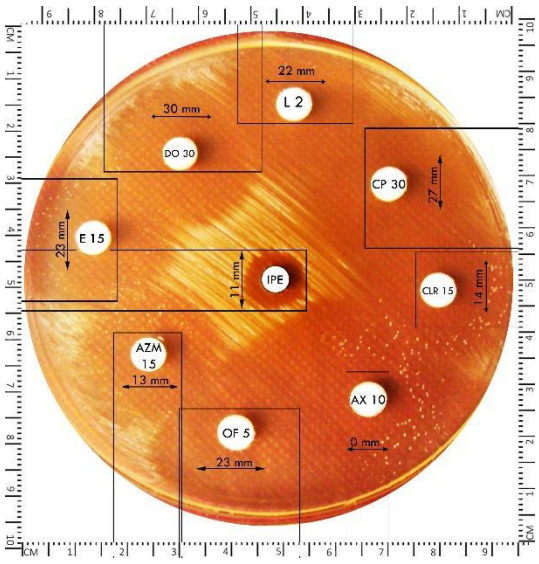
Table.1 Diameter of inhibitions and Degree of sensitive of antibiotics and MEIP in inhibiting growth of *Staphylococcus* spp.

Antibiotic	Diameter of inhibition (mm)	Degree of sensitive
Ciprofioxacin 5µ	25	Sensitive
Norfloxacin 10µ	26	Sensitive
Bactrin 25µ	15	Moderate
Ceftioxone 30µ	12	Moderate
Gentamycin 10µ	13	Moderate
Cefradine 30µ	0	Resistant
Cloxacillin 30µ	0	Resistant
Erythromycin 15µ	23	Sensitive
Doxyciclin 30µ	30	Sensitive
Lincomycin 2µ	22	Sensitive
Offloxacin 5µ	23	Sensitive
Cefalexin 30µ	27	Sensitive
Clarithromycin 15µ	14	Moderate
Azithromycin 15µ	13	Moderate
Amoxycillin 10µ	0	Resistant
Methanol Extract of Indian Propolis (MEIP)	11	Moderate

Fig.1 Effectiveness of antibiotics and MEIP in inhibiting growth of *Staphylococcus* spp



CIP: Ciprofioxacin 5µ, NX: Norfloxacin 10µ, TS: Bactrin 25µ, CRO: Ceftioxone 30µ, GEN: Gentamycin 10µ, CFD: Cefradine 30µ, CX: Cloxacillin 30µ



E: Erythromycin 15µ, DO: Doxyciclin 30µ, L: Lincomycin 2µ, OF: Offloxacin 5µ, CP: Cefalexin 30µ, CLR: Clarithromycin 15µ, AZM: Azithromycin 15µ, AX: Amoxycillin 10µ

Fig.2

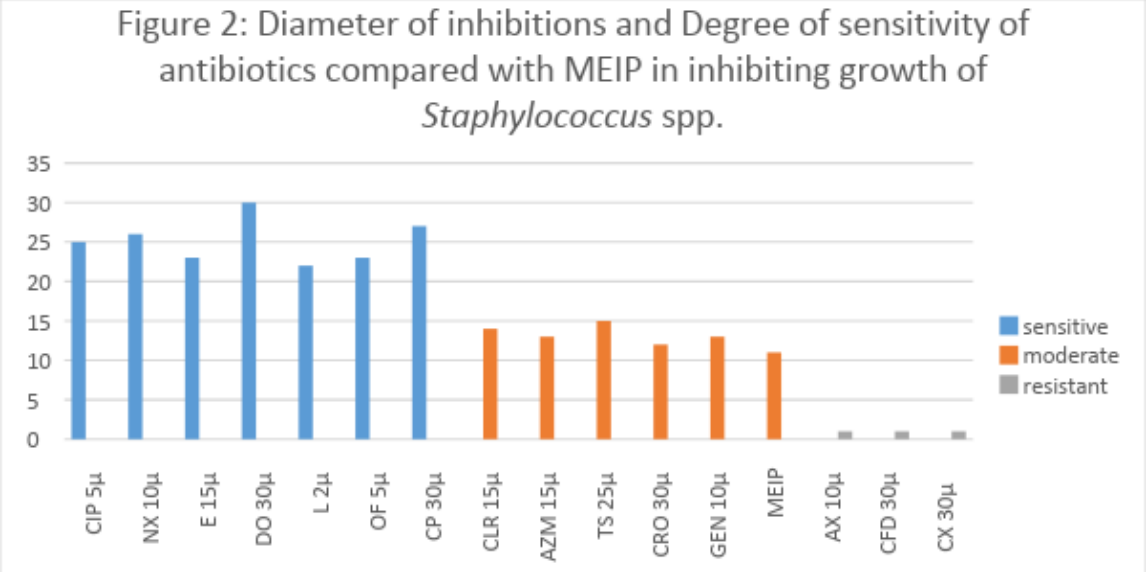
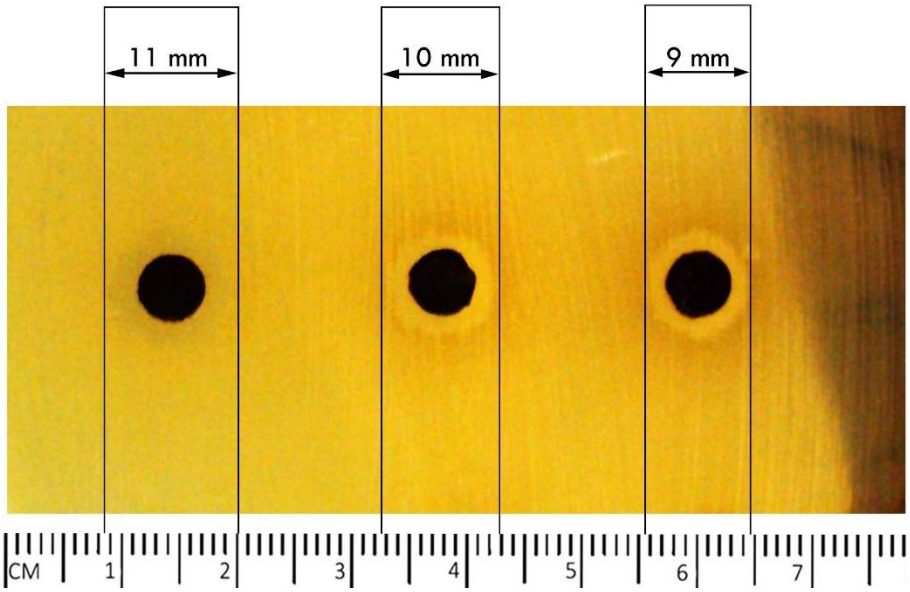


Fig.3 Effectiveness of Indian propolis extraction in inhibiting growth of *Staphylococcus spp.*



It is concluded that *in vitro* test against *Staphylococcus* spp. showed that Ciprofioxacin, Norfloxacin, Erythromycin, Doxyciclin, Lincomycin, Offloxacin and Cefalexin were highly effective against *Staphylococcus* spp. while MIPE showed the similar result with Bactrin, Ceftioxone, Gentamycin, Clarithromycin, and Azithromycin which was moderately effective. Cefradine, Cloxacillin and Amoxicillin were found resistant against *Staphylococcus* spp. The diameter of inhibition growth against *Staphylococcus* spp. was found to be 11 mm with MIPE which was similar to moderate group i.e Bactrin 15mm, Ceftioxone 12mm Gentamycin 13mm, Clarithromycin 14mm, and Azithromycin 13mm. Cefradine 30µ,

Cloxacillin 30µ and Amoxicillin 10µ, considering that they did not show any effect.

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