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ANTIMICROBIAL ACTIVITY OF THE ESSENTIAL OIL EXTRACTED FROM *ARTEMISIA ANNUA*

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ABSTRACT

The present study was undertaken to isolate and identify members of pathogens from milk samples collected from cows showing mastitis and/ or those subjected to antibiotics treatment for prolonged time or any debilitating conditions in Egypt. As well as to evaluate the antimicrobial potential of the extracted oil from *Artemisia Annua* against the recovered microbial species and compare its potency with some of commercially available antibiotics. The obtained results showed that the incidence of isolation of pathogenic strains was 80% out of hundred mastitis cow's milk samples, among those 80 strains; the incidence of *Staphyllococcus aureus* was 26%; of *Streptococcus agalactia* was 20%; *Shigella flexneri* (6%); *Escherichia coli* 15%; *Listeria monocytogenes* 3% and *Candida albicans* 10%. However, the antifungal capacity of the essential oil of *Artemisia annua* has a fungicidal effect on all tested *Candida albicans* strains. Our findings strongly recommend the application of *Artemisia annua* essential oil as a bio-microbicide to reduce the dependence on synthetic pharmaceutical antibiotics and in order to ensure food safety and quality.

KEYWORDS: *Mastitis, Artemisia annua*, Antimicrobial, Resistance, Biofilms**.**

INTRODUCTION

Milk is a rich source of proteins, lipids and sugars, thus, it is considered a tool for many microorganisms to induce serious outbreaks. It contains relatively few bacteria and fungi when it is collected from an udder of a healthy animal, such as Lactobacillus and Candida spp. as they are constitute the most common normal flora of milk **(Baffoni et al., 2012).** However, during milking operations, it gets contaminated from the udder and the adjacent areas, milking utensils, the hands of the milking personnel, from the soil and dust. In these ways bacteria, yeasts and molds get entry into the milk **(Aftab Uddin, et al., 2011).** The World Health Organization (WHO) reported that there are a huge number of human and animal diseases may occur due to the presence of microbes resulting from low or poor hygienic measures and poor sanitation **(WHO, 2002).**

Bacteria usually account for more than 90% of the microbial population in cold raw milk that has been stored **(Garcia Armesto and Sutherland, 1997; Sorhaug and Stepaniak, 1997 and Martins et al., 2006).**

Chapaval et al. (2010) found that release of staphylococcal enterotoxins in milk when milk was stored at temperatures of 37 ºC to 42 ºC or when exposed to variations in temperature.

Similarly, reports have also been made on the incidence of *L. monocytogenes* in milk **(Aurora *et al*. 2006& 2009).** According to several studies, many of the documented outbreaks occurred due to contamination of milk with infectious agents such as Cambylobacter spp**.(Humphrey et al., 2007)*;*** *Salmonella typhi* **(Anderson, 1989);** Streptococcus spp. **(Newsholme, 1902);** *Staphyllococcus aureus* **(Tian et al., 2010);** *E. coli* and *Listeria monocytogenes* **(Corbel 2006).** They are capable to induce different forms of mastitis and subsequently, they are extremely harmful agents for both human and animals; as the enterotoxins produced by some bacterial species are heat – stable, thus, they resist pasteurization and boiling but only autoclaving for 20 minutes can be proper method for complete destroying the toxins **(Dhanashekar et al., 2012).**

Beside, some fungal species such as Nocardia spp., Candida spp. and Cryptococcus spp. can infect the dairy animals as primary causes of clinical, subclinical or chronic mastitis, subsequently they can be excreted in huge amounts in milk **(Streinu-Cercel, 2012).** They are considered opportunistic and their releasing of them in milk produce disease when natural defense mechanisms are lowered and are commonly related to long term and post-treatment

with antimicrobial agents **(Bradley, 2002; Spanammerg et al., 2009; Zaragoza et al., 2011; Guaraldi, 2011 and Arama et al., 2012);** cases with cirrhosis **(Porubcin et al., 2012)** or cases of alcohol consumption **(Solomons, 2012).**

Another critical health – related problem is the microbial resistance that was developed against available pharmaceutical preparations, making microbial drug-resistance a pandemic phenomenon. However, some strains of Candida species are intrinsically resistant to the available antifungal agents **(Rodriguez-Tudela et al., 2008 and Bassetti et al., 2009);** while, others showed an increased biofilm forming ability that influences antifungal susceptibility **(Pulcrano et al., 2012).** In addition, the ability of Candida species to form drug resistant biofilms is an important element in their pathogenicity and inducing microbial drug resistance. Although the correct identification of the infecting organism has become essential for choice effective antifungal therapy, assessing the susceptibility of the identified strains against safe and potent antibiotics is of a great importance **(Kweyang et al., 2012).** These facts enforced the researchers to study the natural plant extracts.

Many studies were conducted on medicinal plant to evaluate their efficacy in order to introduce new medications. One of these plants is *Artemisia annua* (*A. annua*), that plant belongs to the family *Asteraceae*. Former investigations reported that *A. annua* could be used as antibacterial, antiseptic, carminative, digestive, febrifuge and anti-malarial **(Abad et al., 2012; Li et al., 2011; Dehghani et al., 2012; Salih, 2012).**

Therefore, the aim of our study is to isolate the most prevalent pathogens causing mastitis from milk samples in Egypt, assess the *in vitro* activity of *A. annua* essential oil that was extracted by hydro-distillation from the plant on the recovered microorganisms. Beside, the antimicrobial effect of the essential oil (EO) was compared with some commonly used synthetic antimicrobials using disc diffusion test. Calculated minimal inhibitory concentration (MIC) and minimal bacterial concentration (MBC) were also used to evaluate the potency of the essential oil as well.

MATERIALS AND METHODS

Collection of milk samples

A total of one hundred milk samples from subclinical mastitis and apparently healthy cows from different cow's farm in Menufia, Egypt from June to August 2017 were collected under aseptically conditions as per standard -sample collection procedure without giving any stress

on the animals. (Samples were collected from animals after full week of antibiotics stopping) the untreated animals with any antibiotics according to the guidelines of the National Institutes of Health Guide for the care and use of laboratory animals and the General Organization for Veterinary Services in Egypt. The California Mastitis Test (CMT) was used for detection of Sub Clinical Mastitis (SCM) in Bovine milk samples; it was carried out according to Clinical and Laboratory Standard Institute **(CLSI, 2011)** and National Mastitis Council guidelines **(NMC, 2004).** About 2 ml of milk samples from each quarter of dairy Bovine was collected in shallow cups of paddle, an equal amount of CMT reagent was poured into each cup; the sample was mixed by gentle circular rotation, and the results were interpreted based on dense gel formation. About 10-20 ml of milk samples from the teats were collected aseptically after discarding first few streams, in sterile polyethylene screw caped wide mouth vials **(Sharma et al., 2010).** The milk samples were kept in an ice box and carried to the Laboratory of Microbiology Department, Division of Veterinary Medicine, National Research Center (NRC), where the milk samples were kept at 4 – 8^{o}C in refrigerator for further laboratory investigation.

Isolation of bacteria and yeast from milk samples

Refrigerated milk samples which were positive to mastitis test was warmed at room temperature (25°C) for half an hour and then homogenized by gently shaking it in order to disperse bacteria and yeast from milk fat. Bacterial strain isolation from milk samples was carried out following aseptic procedures as described by National Mastitis Council **(NMC, 2004).** A loopful of milk sample was streaked on blood agar (Oxoid) supplemented with 5% sheep red blood cells then the isolates were confirmed by biochemical tests and sub-cultured on differential and selective media, Mannitol Salt Agar, *Salmonella* - *Shigella* Agar and MacConkey Agar (BioMérieux). All plates were then incubated -aerobically at 37°C for 24 h. Identification of the isolates was achieved using Gram's staining, hemolytic pattern, colony morphology, and biochemical tests using Analytical Profile Index (API). Catalase test was applied for distinguishing between *staphylococci* and other Gram-positive cocci, mannitol fermentation test, coagulase test (either positive or negative), bacitracin (0.04 U), furazolidone (100 µg), novobiocin sensitivity. Analytical Profile Index-Staph (API-Staph Kit, bioMerieux, France) was used according to the manufacturer's instructions to differentiate between *Staphylococcus spp*. according to methods described by **López-Malo et al. (2005)** and **Taponen et al. (2006).** Furthermore, Gram-negative bacterial isolation using API-20

tests (API, bio Meraux, France) was carried out according to the standard microbiological procedures **(CLSI, 2006).**

For Yeast, milk samples were centrifuged at 2000 rpm before streaking on Sabouraud's Dextrose Agar with chloramphenicol. The plates were incubated at 37°C for 2-5 days and confirmation was performed depending on morphological characteristics of the yeast by wet mount technique and Gram's stain. Germ tube test was applied on all grown colonies and the positives were identified as *Candida albicans* **(Barnett et al., 1990).** *C. albicans* was further identified by growth at 45°C for observing chlamydospore formation on corn meal agar. All isolates that were positive to primary identification as yeasts were inoculated on CHROM agar and incubated at 37°C for 24 hours and the species were identified by type and colour of the colonies on CHROM agar medium as per manufacturer's instruction **(Lymn et al., 2003).**

Essential oil extraction

The essential oil extracted from *A. annua* was offered by National Institute of Medicinal Plants, Giza, Egypt. A sufficient quantity of the plant (25 gm) in each replicate was added in a round bottom, short necked flask (250 ml. capacity) with sufficient water. The proper essential oil trap and the condenser were attached to the flask, and enough water was added to fill the trap. The flask was placed in an oil bath and heated electrically to approximately 130^{o} C. The temperature of the bath was adjusted so that a condensate of about 1 drop per sec. was obtained. Continue the distillation until no further increase of oil is observed. Usually three hours have been sufficient. When the distillation has been completed, the oil was permitted to stand undisturbed so that a good separation was obtained. The volume obtained was determined and the yield was expressed as a volume/weight percentage; i.e., volume of oil per 100 g of plant herb. The crude oil was dried over pure anhydrous sodium sulfate (120 – 150 g/L of oil). The mixture stored at a temperature of 4^{o} C and was kept in dark and closed bottles to avoid light and oxygen exposure **(Guenther 1972).**

Test of microbial growth inhibition through paper-disc diffusion test

The recovered strains were tested for their antibiotic resistance patterns using the disc diffusion method. Steps of the test and interpretation were relying on the instructions of the National Committee for Clinical Laboratory Standards **(NCCLS, 2002).** The used antibiotic discs (Bioanalyse) were chosen depending on the known patterns of antibiotics against the pathogen and they were: Amoxicillin, 25 mcg AX 25; Tetracycline 30 mcg, TE 30 (for *S. aureus*); Amox./ Clavulanic acid, 20/10 mcg, AMC 30 (for *E. coli*); Ciprofloxacin, 5 mcg,

CIP 5. Clotrimazole 0.01 mg/ml solution (Kripa Pharma) and Fluconazole solution 2 mg/ ml were used to assess the antifungal effect of synthetic agent against the recovered yeast species.

For testing the antimicrobial effect of *Artemisia annua* essential oil we applied the modified technique mentioned by **Desiree *et al*. (2013).** The same selected bacterial and yeast strains were platted on both nutrient and SD agars respectively and incubated at 37°C for 24 h. From each resulted pure culture, microbial suspensions equal to 0.5 McFarland were prepared to obtain the final density required for susceptibility tests by disc diffusion method. The bacterial and yeast suspensions were used to inoculate Muller Hinton agar and Sabouraud's Dextrose Agar respectively, in 90 mm diameter Petri dishes. Two paper discs (6 mm diameter each) were deposited on the preparation then inoculated onto the agar plates. After an hour of incubation at room temperature for all preparations, the cultures plates were incubated at 37°C overnight. Antimicrobial activity for both antibiotics and EO were evaluated by measuring the diameter of inhibition zone around each disc in a monolayer confluent growth. The test was repeated three times and the results obtained were expressed in millimeter. Interpretations were conducted as previously mentioned **(NCCLS, 2002 and Desiree et al., 2013).**

Table (1): The antimicrobials used in the study.

	Organism	**Antibiotics**		**Extracted oil**
1	*Staphyllococcus aureus*	Amoxicillin (AX), 25 mcg	Tetracycline (TE), 30 mcg	Artemisia oil
2	*Escherichia coli*	Amoxicillin (AX), 25 mcg	Amox./ Clavulanic acid (AMC), 20/10	Artemisia oil
3	*Streptococcus agalactia*	Amoxicillin (AX), 25 mcg	Ciprofloxacin (CIP), 5 mcg	Artemisia oil
4	*Listeria monocytogenes*	Amoxicillin (AX), 25 mcg	Ciprofloxacin (CIP), 5 mcg,	Artemisia oil
5	*Shigella flexneri*	Amoxicillin (AX), 25 mcg	Ciprofloxacin (CIP), 5 mcg,	Artemisia oil
6	*Candida albicans*	Fluconazole	Clotrimazole 1%	Artemisia oil

Determination of Minimal Inhibitory Concentration (MIC)

To test the MIC in the present survey, we used the macro-dilution technique performed in liquid medium. In a glass test tube, 200 µL of the EO were added to 2.3 mL of a mixture (0.01% (v/v)) of Mueller Hinton broth and Tween 80 (Loba Chemie PVT). From this original

solution, a serial dilution was performed to have solutions with EO concentrations ranging from 80 to 0.3 mg/ml. To each one, 13 µl of each bacterial and yeast suspensions (0.5 McFarland/100) were added. The set was allowed to incubate aerobically at 37°C (or 30°C) for 24 h. When incubation has completed, all tubes were centrifuged at 5000 rpm for five minutes. The MIC was determined from the first test tube in which no deposit was obtained upon centrifugation. To assess and attest reproducibility, this experiment was also conducted three times **(Hayes and Markovic, 2002).**

Determination of Minimal Bacterial Concentration (MBC)

The solutions from which no deposit was obtained after centrifugation were used to determine the MBC. Briefly, after homogenization, a loop (≈8-10 µL) of each suspension of bacteria and yeast was lawn on MH (and Sabouraud's) agar. This culture was incubated aerobically at 37°C (or 30°C) overnight. The MBC/MFC of the oil was inferred from the culture medium in which no visible microbial growth was recorded upon revelation. This experiment was repeated three times too **(Hayes and Markovic, 2002).**

RESULTS AND DISCUSSION

Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder **(Toll, 1980)**. Beyond this stage of milk production, microbial contamination can generally occur from three main sources **(Bramley and McKinnon, 1990);** from the udder, from the exterior of the udder and from the surface of milk handling and storage utensils. Bacterial contamination of raw milk can originate from different sources: air, milking equipment, feed, soil, feces and grass **(Coorevits et al., 2008).**

The prevalence of bacterial and yeasts strains were mentioned (Table 2), the highest percentage of isolation was for *S. aureus* (26%) followed by *S. agalactia* (20%), while the lowest prevalence was recorded for *L. monocytogenes* (3%) followed by *Sh. flexneri* (6%). These results indicated the strong relation between incidence of some pathogens as contaminants and the poor sanitary conditions applied before and during milking process; while the isolation of *S. agalactia*, *L. monocytogenes*, *Sh. flexneri* and *C. albicans*, the pathogenic species among the candida species, indicating that these latent pathogens are the causative agents of mastitis and cannot be considered as normal flora or contaminants. In general, the incidence of candidiasis caused by Candida spp. was reported to indicate the increase in proportion to the growing number of immune-compromised, cancer and postoperative patients. While, antibiotics promote yeast infections, including gastrointestinal

Candida spp. overgrowth, and invasion of the gastrointestinal mucosa **(AL-Abeid et al., 2004).** In addition, there are other predisposing factors for susceptibility to yeast infections and Candidiasis as prolonged antibiotic therapy and diabetes or impairment of immune systems **(Kennedy, 1987)**.

Table (2): Isolation of pathogenic bacteria and yeasts from mastitic milk samples.

Isolated Strains	No. of isolated strains	% of isolation
Staphyllococcus aureus	26	(26.0%)
Streptococcus agalactia	20	(20.0%)
Shigella flexneri	6	(6.0%)
Escherichia coli	15	(15.0%)
Listeria monocytogenes	3	(3.0%)
Candida albicans	10	(10.0%)
Total	80	(80%)

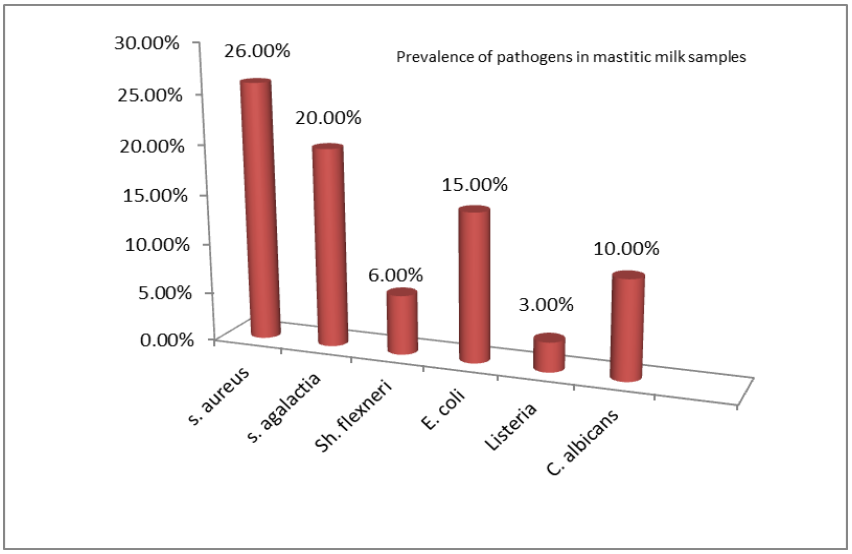

Fig. 1: Types and prevalence of pathogenic strains isolated from mastitic milk.

Microbial growth inhibition

The antimicrobials used in the current study were chosen according to the documented data of each antibiotic against each recovered microbe, thus, the inhibitory effect of essential oil of *A. annua* was compared with antibiotics having strong inhibitory effect from one side and with those antibiotics usually show resistance by the tested pathogens. This resistance that may lead to weak response to antibiotics or even lead to chronic mastitis and/ or mycosis.

The potency of the essential oil extracted from *A. annua* was nearly equal to that of Amoxicillin against *S. aureus*, *S. agalactia*, *L. monocytogenes* and *C. albicans*, while this

potency was less than that of Amoxicillin in cases of *E. coli* as depicted in table (3). Similar results were reported previously by **Desiree et al. (2013)**, **Juteau et al. (2002) and Verdian – Rizi et al. (2008).**

The weak inhibitory effect of EO against *Sh. flexneri* revealed by our results (3 mm ± 0.5) was similar to that mentioned by **Desiree et al. (2013).** In fact, *Sh. flexneri* showed resistance not only against EO, but against Ciprofloxacin too, revealing one of the most serious disadvantageous resulting from antibiotics abuse.

Table (3): Inhibition diameter of antimicrobials compared with *A. annua* oil.

Organism	Antibiotics	Inhibitory zone Ø	Reference Interpretation
Staphyllococcus aureus	Amoxicillin (AX), 25 mcg	20mm ± 0.5	S: (20mm)
	Tetracycline (TE), 30 mcg	14mm ± 0.0	R: (<11mm)
	Artemisia oil	20mm ± 0.0	S: (>15mm)
Escherichia coli	Amoxicillin (AX), 25 mcg	17mm ± 0.0	S: (20mm)
	Amox./ Clavulanic acid (AMC), 20/10	10mm ± 1.0	R: (<13mm)
	Artemisia oil	12mm ± 0.0	*I: (S >15mm)
Streptococcus agalactia	Amoxicillin (AX), 25 mcg	16mm ± 0.5	S: (20mm)
	Ciprofloxacin (CIP), 5 mcg,	9mm ± 0.5	I: (16-20mm)
	Artemisia oil	15mm ± 0.5	S: (>15mm)
Listeria monocytogenes	Amoxicillin (AX), 25 mcg	22mm ± 1.0	S: (20mm)
	Ciprofloxacin (CIP), 5 mcg,	5mm ± 3.0	R: (<15mm)
	Artemisia oil	18mm ± 1.0	S: (>15mm)
Shigella flexneri	Amoxicillin (AX), 25 mcg	19mm ± 0.5	S: (20mm)
	Ciprofloxacin (CIP), 5 mcg,	5mm ± 2.0	R: (<15mm)
	Artemisia oil	3 mm ± 0.5	*R: (S>15mm)
Candida albicans	Fluconazole	18 mm ± 0.5	S: (>15mm)
	Clotrimazole 1%	30 mm± 0.5	S: (>15mm)
	Artemisia oil	23 mm ± 1	S: (>15mm)

Many studies indicated that the pathogen – host immune system interaction would never been clarified and the response of pathogen towards a certain antimicrobial is greatly varies depending on many factors most of them are related to the host immune system. Thus, it is not necessary for an organism to have the same pattern in case of microbial infection (*in vivo*).

Minimal Inhibitory Concentration (MIC) & Minimal Bactericidal Concentration (MBC)

Figure (2) shows that, except for *Sh. flexneri*, the minimal inhibitory and lethal concentrations (bactericidal and fungicidal) were nearly equal (MIC /MLC = 1) for all the tested strains. For *Sh. flexneri*, there wasn't a concentration within the prepared range could inhibit the growth of *Sh. flexneri* (MIC was higher than the maximum level 80 mg/mL). *C. albicans* was the most susceptible isolate to EO (MIC and MBC = 10 mg/mL) while the highest MIC and MBC were obtained with *S. agalactia* and *L. monocytogenes.* For the other, the MIC and MBC values were lower than 20 mg/ml. These findings are consistent with those obtained in disc-diffusion test as discussed above.

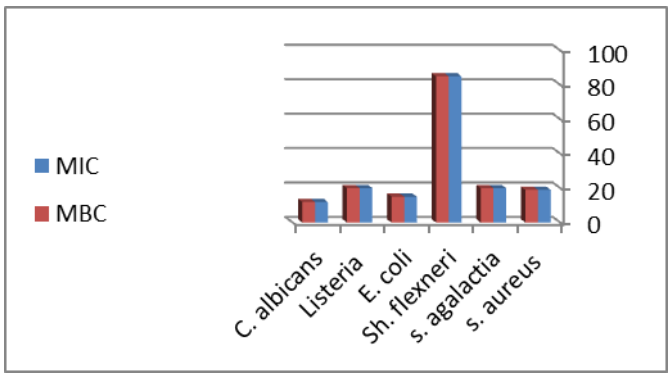

Fig. 2: Minimal Inhibitory Concentration (MIC) & Minimal Bacterial Concentration (MBC).

Essential oil of *A. annua* contains the following chemical compounds: camphor (major component), α-pinene, ß-pinene, 3-carene, α-terpinene, artemisia ketone, copaene, camphene, caryophylene, menthol, α-terpineol. Beside, Benzyle 2-methyl butyrate, Tranc-Caryophyllene, β- farnesene and β- Selinene are most probably having antimicrobial effect **(Li et al., 2011)**, however, it cannot be confirmed which of these components are responsible for the microbicidal properties of the oil. Hence, further investigations should be conducted to study the antimicrobial activity of whole plant extract and its oil as well as to determine the active components of the oil and to define the *in vivo* antimicrobial potentials of the oil's components on laboratory animal.

CONCLUSIONS

In view of all the above, essential oil of Artemisia annua is considered a potent, active and safe natural agent can be used as microbicidal herbal agent. Further studies must be

undertaken to evaluated field application of such oil against wide range of bacterial and fungal species for controlling most of microbial diseases.

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