Wayamba Journal of Animal Science – ISSN: 2012-578X; P582-P589, 2013
First Submitted March 13, 2013; Number 1363148715

Curcuma longa: AN ALTERNATIVE TO ANTIBIOTICS TO COMBAT MASTITS IN CATTLE

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This study investigated the effect of various antibiotics and the raw extract of plant *Curcuma longa* (Haldi) against micro-organisms responsible for bovine mastitis-inflammation in the mammary gland. Milk was collected from the affected cows from Anand district in Gujarat. Microorganisms from the milk were isolated and characterized using various biochemical tests. In vitro antimicrobial susceptibility pattern of all the isolates was checked against 16 different antibiotics using the agar disc diffusion method. Of the 12 isolates selected in the study, most showed total resistance to Gentamicin and Erythromycin antibiotics. It was also observed that the patterns of bacterial resistance have not changed in India over the years. *C. longa* raw extracts from its roots were prepared in ethanol and tested against the mastitis causing organisms by adding its different concentrations in wells prepared in the agar plates. The result shows significant antibacterial activity against the tested mastitis causing bacteria. However the effect was checked on a few elite organisms. Promising effect of *C. longa* in combating mastitis pathogens incites further research in the area.

Keywords: Antimicrobial susceptibility, mastitis, *Curcuma longa,* agar disc diffusion

Mastitis is the inflammation in the mammary gland of the cattle owing mainly to microbial infections and, it is the most serious and economically important disease in dairy milk production worldwide [1-5]. Mastitis-related losses are associated with reduction in yield, increased treatment costs, discarded milk, increase in culling and associated dairy cow replacement rates, and financial penalties for exceeding legal milk quality limits [6, 7]. The main etiological agents responsible for this disease can be divided into several groups of organisms like contagious pathogens, environmental bacteria, opportunistic bacteria, mycoplasma, fungi, and algae-bacteria being the most frequent contributed especially by the golden *Staphylococcus* [8-13].

Current treatment options and their drawbacks

Current treatment to this disease mainly relies on administering antibiotics to the cattle. However, the biggest threat with antimicrobials is resistance development amongst the microbial community [14, 15]. Many bacteria develop resistance mechanisms, enabling them to inactivate antimicrobial compounds in their environment. Genetic exchange between similar or different bacterial species may result in the spread of resistance genes [16]. Pathogens in animals that are used for food products pose one of the greatest risks for human health, as this is a major route for the transfer of bacteria from animals to humans [17, 18].The incorrect use of antibiotics in the treatment of diseases such as mastitis and for use in feed as growth promoters has led to the assumption that antibiotic resistance in bacteria could become more widespread because of the transmission of resistant zoonotic and non-zoonotic bacteria via food. This could have a serious impact

on the treatment of bacterial pathogens causing disease in humans if similar antibiotics are used [19, 20]. Pathogens associated with mastitis can also infect humans, e.g. food poisoning by *Staphylococcus aureus* another mastitis causing pathogen *Streptococcus agalactiae* causes septicemia and neonatal meningitis [21, 22].

Also from a commercial standpoint, milk products containing specific levels of antibiotic residues cannot be sold for human consumption [23]. Processing of cheese and yoghurt manufacture is also affected as bacterial starter cultures are inhibited and the quality of the product produced is generally compromised [24]. Completely eliminating the use of antibiotic from the treatment of mastitis is also unlikely as high demand of milk dictates rapid and intensive treatment strategies. Thus, antibiotics are required to be administered in a manner so as to protect and prevent the spread of the disease, in addition, the human food safety must also be ensured. Antibiotics have been used for many years to eliminate bacterial pathogens causing disease in the case of mastitis it is important to note that antibiotic therapy cannot be relied upon to reduce the incidence of mastitis as a standalone anti mastitis action. The ultimate goal thus would be to reduce the use of antibiotics and search for a better and effective alternative.

Plants: an alternative to antibiotic therapy

Clinical microbiologists and veterinarians have two reasons to be interested in the topic of antimicrobial plant extracts. Firstly, it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians. Secondly, herbal products are readily available over –the-counter from herbal suppliers and natural food stores.

Extracts from *Curcuma longa* has been shown to have anticancer, anti-inflammatory, anti-oxidative properties [25]. Curcumin is a yellow orange powder that is insoluble in water and ether but soluble in ethanol, dimethylsulfoxide and acetone.

The present study aimed to develop an antibiogram against the mastitis causing organisms and test the antimicrobial effect of crude extract (ethanolic) of *Curcuma longa* (Haldi) against the microbes causing mastitis and attempt to search the better option of the two.

MATERIALS AND METHODS

Collection of Milk sample

Milk samples were collected from cows diagnosed with clinical mastitis (in and around Anand city of Gujarat state). Clinical mastitis was confirmed by the physical examination of udder, symptoms, history, etc.

Microbiological and biochemical characterization

Milk samples from affected cows, were initially plated on defibrinated 5% sheep blood agar (SBA) plates (and incubated at 37 ^{o}C for 24–48 h) for isolation. Tests for identification of the microorganisms were performed. Macroscopic characteristics of the colonies on nutrient agar, production or absence of hemolysis, Gram staining, catalase test, coagulase test and other biochemical tests were performed. Twelve colonies showing distinct alpha, beta or gamma hemolysis and distinct morphologies on nutrient agar were selected for antimicrobial studies. Pure colonies were obtained after 2 subsequent streaking on nutrient agar plates.

In Vitro antimicrobial susceptibility testing

Antimicrobial susceptibility patterns of all the isolates were checked using agar disc diffusion method against 16 different antibiotics. The isolates were pre-cultured in nutrient broth in a rotary shaker at 37 ^{o}C, centrifuged at 10,000 rpm for 5 min, pellet was resuspended in double distilled water and the cell density was standardized spectrophotometrically (A_{610} nm). The test microorganisms were then seeded into nutrient agar plates by spread plate method 10 µL (10^{6} cells/mL). Antibiotic discs were then placed on the bacterial lawn with the help of sterile forceps and incubated at 37 ^{o}C for overnight. The sensitivity was measured in terms of the diameter of the zone of inhibition surrounding the disc.

Collection of plant material

Fresh, healthy roots of *Curcuma longa* were collected from surroundings of Vallabh Vidhyanagar of Gujarat state. The roots were washed thoroughly with running water and once with sterile distilled water and then air-dried on sterile blotter under shade.

Preparation of extract

The method of Saxena *et al*. (1993) was adopted for the preparation of plant extract [26]. Healthy roots/fingers of the plant were thoroughly washed with running tap water blotted and shade dried till they became crisp. For the purpose of making powder it was grinded in the grinder (Maharaja Mixer Ltd.). Powder obtained (around 20 gm) was soaked in 100 mL of ethanol for 24 h at room temperature under shaking conditions (130-160 rpm). The extract was then filtered with the help of Whatman filter paper 1. The filtrate was collected in petridish and dried at 50 ^{0}C. The dried extract was then scraped and transferred into eppendorf tubes.

The residual material from the funnel was dried again and resuspended in 100 mL of ethanol for 24 h at room temperature under shaking conditions (130-160 rpm). The crude extract was filtered and collected in petridish and the same procedure was repeated. The collected extract was then weighed and stored at 4 ^{o}C until further use. No further fractionation or characterization was done as the objective was to test the crude extract itself.

Preparation of various concentration solutions of the plant extract

Four different concentrations were prepared to determine the Minimum inhibitory concentration (MIC) (Pelczar *et al*., 1986), 5, 25, 50 and 100 mg/mL in ethanol [27]. These were filter sterilized and used.

Well diffusion method

The agar well diffusion method [28-31] was followed. Using a sterile cotton swab lawn cultures of the test organisms were grown on the nutrient agar plates. Five mm diameter was punched into the agar on each plate using a sterile well cutter. Into each well 30 µl of the plant extract was added. The solution was allowed to diffuse for 2 h by placing the plate in refrigerator. The plates were incubated at 37 ºC for 24-48 h. The antibacterial activity was evaluated by measuring the zone of inhibition around the well. The zone of inhibition (excluding well diameter) was measured as a property of antibacterial activity. Antibiotic Gentamicin (10 mcg) was used as positive control and ethanol was used as negative control.

RESULTS

Different biochemical tests carried out on the 12 isolates were shown in Table 1. Twenty three biochemical tests including the Gram's staining (data not shown) were performed in order to identify the unknown bacteria. Some of the tests provided immediate results while others had to be incubated for a period of time. A Phenol red test looks at the bacteria's ability to ferment glucose, lactose, or sucrose with the help of a pH indicator. A Durham tube was also placed in the tube to collect any CO_2 that might have been produced [32]. All the 12 isolates were found to utilize glucose sucrose lactose and mannitol as carbon source. Triple sugar iron (TSI) agar tube was used to test for the fermentation glucose, lactose and sucrose by production of ferrous ammonium sulfate [32]. All the 12 isolates were found to be lactose fermenters as supported by the acidic changes in the medium as detected by the presence of yellow color throughout the medium in test tubes. Five out of 12 isolates were found to hydrolyze starch. Only one isolate was found to be positive for indol test. Methyl red test was found positive only in one isolate whereas 4 isolates were found positive for VP test. None of the 12 isolates were found to be positive for citrate tests. All the isolates were found positive for catalase tests except two. Only one isolate was found to be showing the alpha hemolysis in hemolysin test and three were showing beta hemolysis while the rest all showing gamma hemolysis. Coagulase test was carried out to specifically identify the *Staphylococcus* species carrying the coagulase gene.

Table 2 shows the antimicrobial susceptibility testing results performed on the 12 isolates with 16 antibiotics. All the 12

Table 1: *Biochemical tests performed on all the isolates (1-12).*

Biochemical Tests				**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Utilization of carbohyarates and organic acids	*Sugar fermentation test*	Glucose		+	+	+	+	+	+	+	+	+	+	+	+
		Sucrose		+	+	+	+	+	+	+	+	+	+	+	+
		Lactose		+	+	+	+	+	+	+	+	+	+	+	+
		Fructose		+	+	-	-	+	+	+	+	-	+	+	-
		Mannitol		+	+	+	+	+	+	+	+	+	+	+	+
	Oxidation-fermentation test	*Tubes with paraffin oil*	*Acid production*	-	+	-	-	-	-	-	+	-	-	+	+
			Gas production	-	-	-	-	-	-	-	-	-	-	-	-
		Tubes without paraffin oil	*Acid production*	-	+	-	-	-	-	-	+	-	-	+	+
			Gas production	-	-	-	-	-	-	-	-	-	-	-	-
		Type of metabolism		N	F	N	N	N	N	N	F	N	N	F	F
	Methyl red test			-	+	-	-	-	-	-	-	-	-	-	-
	Voges-Proskauer test			+	-	-	-	-	-	+	+	+	-	+	-
	Citrate utilization test			-	-	-	-	-	-	+	-	-	+	-	-
Utilization of Nitrogenous compounds	*Indole production test*			-	+	-	-	-	-	-	-	-	-	-	-
	Hydrogen sulphide production test(lead acetate paper strip test)			+	-	-	+	-	-	-	+	-	-	-	-
	Phenylalanine deamination test			-	-	-	-	-	-	-	-	-	-	-	-
	Urea hydrolysis test			-	-	-	-	-	-	-	-	-	-	-	-
	Nitrate reduction test			+	+	+	+	+	+	+	+	+	+	+	+
	Ammonia production test			-	+	+	-	+	+	+	+	-	+	+	-
Decomposition of large molecules	*Starch hydrolysis test*			-	-	-	-	+	+	+	-	-	-	+	+
	Gelatin hydrolysis test			-	-	-	-	+	+	+	+	-	-	+	+
	Casein hydrolysis test			-	-	-	-	-	+	-	-	-	-	+	+
Miscellaneous tests	*Catalase test*			+	-	+	-	+	+	+	+	+	-	+	+
	Dehydrogenase test			-	-	-	-	-	-	-	-	-	-	-	-
	Hemolysin production test			A	Ɣ	β	Ɣ	Ɣ	Ɣ	A	Ɣ	β	Ɣ	Ɣ	β
	Coagulase test			-	-	+	-	-	-	-	-	+	-	-	-
Combine tests using composite media	*Triple sugar iron agar test*	*Acid*		+	+	+	+	+	+	+	+	+	+	+	+
		Alkaline		-	-	-	-	-	-	-	-	-	-	-	-
		Gas		-	-	+	-	-	-	-	-	-	-	-	-
		Lactose fermenter		+	+	+	+	+	+	+	+	+	+	+	+

'+' shows positive result and '-' shows negative result, N=non fermentative metabolism, F=fermentative metabolism, α=Alpha hemolysis, β=beta hemolysis, Ɣ=gamma hemolysis.

Table 2: Antibiotyping of different antibiotics on lab isolates

No.	Antibiotic	Concentration (mcg)	Lab Isolates											
			1	2	3	4	5	6	7	8	9	10	11	12
1.	*Amikacin*	10	+	+	+	+	+	+	+	+	+	+	-	+
2.	*Ampicillin*	10	+	+	+	+	+	+	+	+	-	-	+	+
3.	*Amoxicillin*	10	-	+	-	+	+	+	+	+	+	-	+	+
4.	*Chloramphenicol*	10	+	+	-	+	+	+	+	+	+	+	-	+
5.	*Erythromycin*	15	+	+	+	+	+	+	+	+	+	+	+	+
6.	*Gentamycin*	10	+	+	+	+	+	+	+	+	+	+	+	+
7.	*Kanamycin*	5	+	+	-	+	+	+	+	+	+	-	+	+
8.	*Lincomycin*	10	-	+	-	+	+	-	+	+	-	-	+	+
9.	*Nalidixic acid*	30	-	+	+	+	+	+	+	+	+	+	-	+
10.	*Oxacillin*	1	-	+	-	-	+	+	+	+	-	-	-	-
11.	*Oxytetracyclin*	30	+	+	+	+	+	+	+	+	+	-	+	+
12.	*Penicillin*	10	-	+	-	-	+	+	+	+	-	-	-	-
13.	*Rifampicin*	5	+	+	-	+	+	+	+	+	+	+	+	+
14.	*Streptomycin*	10	+	+	+	+	+	+	+	+	+	-	+	+
15.	*Tetracyclin*	30	+	+	-	+	+	+	+	+	+	+	+	+
16.	*Trimethoprin*	5	-	+	-	-	+	+	+	+	+	-	+	+

'+' shows the formation of zone of inhibition,'-' shows absence of zone of inhibition.

Table 3: Antimicrobial activity of Curcuma longa extract

Culture isolates	**Ethanol (Cm)**	**5mg/mL (Cm)**	**25mg/mL (Cm)**	**50mg/mL (Cm)**	**100mg/mL (Cm)**	**10 mcg Gentamicin (Cm)**
1	0.2	0.8	1.0	1.12	1.12	3.2
2	0.2	0.7	0.8	0.8	0.8	0.6
3	0.2	0.9	0.9	0.9	1.5	0.6
4	0.4	0.9	0.9	0.9	1.8	0.6
5	0.2	0.8	0.8	1.0	1.7	1.0
6	0.2	0.7	0.9	1.2	1.8	2.8
7	0.3	0.7	0.7	0.7	2.0	0.4
8	0.2	0.5	0.8	0.8	1.0	1.7
9	0.4	1.3	0.9	0.7	0.7	1.2
10	0.1	0.5	0.7	0.8	0.7	2.0
11	0.1	0.7	0.7	0.8	1.0	0.6
12	N	0.7	0.7	0.7	0.8	2.3

Values are mean inhibition zone (cm) of three replicates

isolates were found to be susceptible to Gentamicin and Erythromycin at the concentrations used 10 mcg and 15 mcg, respectively. Isolate 10 and 3 were found to be resistant to 9 antibiotics (both were resistant to antibiotics-Amoxicillin, Penicillin, Kanamycin, Oxacillin and Trimethoprim) out of 16, turning out to be the most resistive isolates of the 12 isolates.

Results of the antibacterial activity of raw extract of *C. longa* are shown in Table 3. Ethanol was used as the negative control and antibiotic Gentamycin was used as the positive control. The amount of extract obtained by dissolving 20 gm grinded powder of *C. longa* in 100 mL methanol as solvent was 1.77 gm. *C. longa* extract showed prominent antibacterial activity at tested concentrations against isolates 1, 4, 5, 6 and 7 as compared to other isolates. Highest activity (2.05cm) was seen at concentration of 100 mg/mL of the extract against the isolate 7 and lowest activity (0.5cm) was seen at 5 mg/mL concentration against isolates 8 and 10. In some cases similar antimicrobial activity (0.7cm) was seen at two different concentrations (mainly 5 mg/mL and 25 mg/mL).

DISCUSSION

The macroscopically distinct colonies were first characterized for their Gram's staining (data not shown) and then various biochemical tests were performed on 12 isolates in order to characterize them broadly at least at the species level. Few tests served to identify the *Staphylococcus* and *Pseudomonas* species, like the hemolysis tests-generally the *S. aureus* and *Pseudomonas aeruginosa* strains show β hemolysis on blood agar plates. β-hemolysis is a complete lysis of red cells in the media around and under the colonies, the area appears lightened and transparent. *Streptococcus* strains like *Streptococcus pneumoniae* tend to show alpha hemolysis (partial hemolysis with a greenish discoloration of the blood agar surrounding a bacterial colony). A positive Indole red, methyl red, nitrate reduction and ammonia production and a negative gelatin, starch hydrolysis, citrate utilization and urease test suggest the bacterial isolate to be *Escherichia coli*. To further identify the *Staphylococcus* species the strains were streaked on selective medium like mannitol salt agar, *S. aureus* species turn the agar from red to yellow while the *Staphylococcus epidermidis* does not change the medium. The strains were also streaked on potassium telurite agar, *Staphylococcus* and *Corynebacterium* species reduce the potassium telurite in agar and grow as black colored colonies. Coagulase test served to differentiate between the *Staphylococcus* species as coagulse is produced by *Staphylococcus* strains like *S. aureus*. Overall *Staphylococcus* (isolate 3, 9 and 12), *Streptococcus* (isolate 1 and 7), *E. coli* (isolate 2), *Corynebacterium* and *Bacilli* genus of bacteria could be identified from the tests. The genus can be confirmed by 16S rDNA amplification and sequencing of the isolates and they can be identified at the species level thereby.

C. longa is considered to have antimicrobial properties and is used as an antiseptic to cure wounds, cuts and other infections, because of this beneficial property an attempt was made to study whether same type of potential effect is seen on mastitis causing microorganisms or not, positive results will help in lowering the incidences of intramammary infections by pathogens. Use of frequent antibotics against the microbes poses the risk of development of resistance in the microbial community. From our results it is seen that raw extract of *C. longa* can be useful to combat a few of the mastitis causing pathogens especially the *Streptococcus* and *Staphylococcus* species. In some of the cases higher concentrations did not show prominent increase in the zone of inhibition and hence the activity. The type and level of biological activity exhibited by any plant material depends on many factors, including the plant part, geographical source, soil conditions, harvest time, moisture content, drying method, storage conditions, and post-harvest processing etc. In this study ethanol was chosen as extraction solvent because ethanol formulations are relatively safe for human and animal consumption as compared with other organic solvents, such as acetone or methanol frequently used by researchers. Further, ethanol extraction is widely used to obtain crude extracts of phytochemicals from plant materials in the herbal medicine industry for therapeutic applications. Due to the variation in composition of active compounds, different plant types may require different concentrations of ethanol to achieve maximum recovery of bioactive components. No standardized extraction protocol has been developed for preparation of herbal extracts, but 20-95% of ethanol-water mixture is frequently used by the herbal medicine industry to prepare ethanolic extracts [30]. In the present study, we used 50% of ethanol in water and at this tested concentration an average of 0.2 cm antibacterial activity was observed. In some of the cases such as the isolates (1, 6, 8, 10 and 12) antibiotic Gentamicin used as control showed better activity as compared to the *C. longa* extract. Whereas in isolates 3, 4, 5 and 7, *C. longa* extract at concentration of 100 mg/mL showed prominent antimicrobial activity at an approximate average of 1.7 cm of zone of inhibition. A few isolates showed same susceptibility to different concentrations of the *C. longa* ethanolic extract. This incites

further research in the study as the reason for this inhibition is still unknown. A number of studies have voiced the necessity of developing alternative antimicrobial drugs [33, 34]. Plant antimicrobials would appear to be an excellent choice [35]. However, antibiotics are still necessary to combat bacterial pathogens as is shown by the antibiotic susceptibility of pathogens towards Gentamicin which in some cases is higher than the susceptibility towards *C. longa* and hence, a solution could be to focus research into the development of other antimicrobial agents that offer some advantages over the antibiotics that are currently in use.

In conclusion antibiotyping studies report that the growth of all the organisms is inhibited by Erythromycin and Gentamicin antibiotics, while the rest of them showed varied effects on different pathogens isolated, a few resistant species were also detected. In case of *C. longa* extract a few positive results can elite further research in this area to search Herbal/natural alternative for mastitis treatment. Combination of both the treatment options could be more effective against this disease.

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