Haryana Vet. (March, 2020) **59(SI),** 1-9 **Review Article**

ESSENTIAL OILS AND MASTITIS IN DAIRY ANIMALS: A REVIEW

RENU GUPTA*, SANDEEP KUMAR[1] and RAJESH KHURANA
Department of Veterinary Public Health and Epidemiology, [1]Department of Veterinary Physiology and Biochemistry, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125 004, India

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ABSTRACT

Treatment of mastitis in dairy animals is invariably carried out using antibiotics and ever increasing problem of antimicrobial resistance has increased the risk of relapse. Various therapeutics have been documented by various workers including medicinal plants. Herbal formulations have a mixture of various phytochemicals including essential oils. Therefore, through this review, attempts have been made to analyze various publications reporting findings on use of essential oils and/or their components in relation to mastitis in domestic animals. There are many *in vitro* studies for evaluating the efficacy of essential oils for their antimicrobial action against many isolates of mastitic origin and many essential oils and their components have been found to be effective with a wide range of MIC and MBC. Contrarily, there are only a few *in vivo* studies to assess their therapeutic effects. Also, issues have been raised about their use as therapeutic agents as the required high concentration will be too irritating and may cause damage to mammary tissues. Further clinical trials are warranted to determine safety and possible withdrawal times in milk before its recommendation for use in organic operations. Owing to their wonderful antimicrobial activity, if their tissue irritant properties can be mitigated by some manipulations, they may be of immense help in fighting mastitis in future.

Keywords : Essential oil, *In vitro*, Inramammary, Mastitis, Minimum inhibitory concentration

Mastitis, as the name suggests is an inflammatory condition of the mammary gland. This is one of the most significant diseases affecting lactating animals leading to huge economic losses (Aghamohammadi *et al.*, 2018). Mastitis due to infectious agents exists in clinical and subclinical forms. Treatment of infections is invariably carried out using antibiotics and ever increasing problem of antimicrobial resistance has increased the risk of relapse especially when a bacteriological cure is not achieved (Gomes and Henriques, 2016).

The public health concern about milk is of being it an important source of antimicrobial drugs and resistant microorganisms which enter the human food chain (Kromker and Leimbach, 2017). Therefore, there is utmost need to identify and use alternatives to antibiotics to prevent and treat mastitis in food-producing species. Various therapeutics have been documented by various workers including medicinal plants (Mushtaq *et al.*, 2018), homoeopathy (Zeise and Fritz, 2019), bacterocins (ribosomally synthesized polypeptides) (Godoy-Santos *et al.*, 2019), bacteriophages, nanoparticles (Gomes and Henriques, 2016) and gut microbes (Pellegrino *et al.*, 2019) among others. Other less worked methods include hydrotherapy (Duval, 1997), clay therapy, acupuncture (Duval, 1997) and acoustic therapy (Leitner *et al.*, 2018). Long lasting reactive species (LARS) by Westway Health, Ireland and therapy using Electrical signals by Prise Systems, Israel are the latest alternatives that are claimed to be highly promising for treating mastitis (Willmer, 2019).

Herbal formulations have a mixture of various phytochemicals. The cocktail of various bio-molecules target multiple receptors and the resulting complex interaction is important for resulting healing processes. Various types of herbal formulations used for therapeutics include whole or specifics parts of plants, crude extracts, ethyl acetate extract, alcoholic extracts, alkaloids, flavonoids, saponins, fixed oils, essential oils, lipid rich extracts and purified phytochemicals (Spinella, 2002).

Corresponding author: renu.gupta@luvas.edu.in

Essential oils as defined by The International Organization for Standardization (ISO) are "product obtained from a natural raw material of plant origin, by steam distillation, by mechanical processes from the epicarp of citrus fruits, or by dry distillation, after separation of the aqueous phase- if any- by physical processes". Almost 3000 different essential oils have been described (Burt, 2004). These oils are being used in medicine, cosmetics, food and as dietary supplement. Medicinal uses of oils include as anti-inflammatory, wound healer, relaxant, analgesic, antimicrobial, antiviral, immune strengthener, digestive aid etc. (Manion and Widder, 2017). Going by various medicinal properties of essential oils especially their antimicrobial activity, the present review attempts to analyze various publications reporting findings in relation to mastitis (prevention, treatment and *in vitro* research) in domestic animals.

METHODOLOGY

Publications were searched through The National Center for Biotechnology Information (NCBI) database and Google search engine. Relevant publications were selected and analyzed. Various studies that have been reviewed for this study have been summarized in Table 1.

Table 1

Various studies included in the review

Study	*In vivo/ In vitro*	Country of work
Abdalhamed *et al*., 2018	*In vitro*	Egypt
Alekish *et al*., 2017	Both	Jordan
Baskaran *et al*., 2009	*In vitro*	USA
Budri *et al*., 2015	*In vitro*	Brazil
Castro *et al*., 2016	*In vitro*	Brazil
Cerioli *et al*., 2018	*In vitro*	Argentina
Cho *et al*., 2015	*In vivo*	Korea
Choi *et al*., 2012	*In vitro*	Korea
Cuc *et al*., 2010	*In vitro*	Romania
Dal Pozzo *et al*., 2011	*In vitro*	Brazil
Dal Pozzo *et al*., 2012	*In vitro*	Brazil
Federman *et al*., 2016	*In vitro*	USA
Fratini *et al*., 2014	*In vitro*	Italy
Grzesiak *et al*., 2016	*In vitro*	Poland
Grzesiak *et al*., 2018	*In vitro*	Poland
Hase *et al*., 2013	*In vivo*	India
Ksouri *et al*., 2017	*In vitro*	Algeria
Lefevre *et al*., 2008	Both	France
Montironi *et al*., 2016	*In vitro*	Argentina
Montironi *et al*., 2019	*In vivo*	Argentina
Mullen *et al*., 2014, Mullen, 2020	*In vitro*	USA
Noori *et al*., 2019	*In vitro*	Iran
Norby and Halbert (2014)	*In vitro*	USA
Piotr *et al*., 2018	*In vitro*	Poland
Queiroga *et al*., 2018	*In vitro*	Portugal
Soares *et al*., 2013	*In vitro* (Patent for product for *in vivo* application)	Brazil
Tortorano *et al*., 2008	*In vitro*	Italy

The review is broadly categorized into *In vivo* and *In vitro* studies. *In vitro* studies are further reviewed on the basis of type of study.

IN VITRO STUDIES

I. Antibacterial activity in terms of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Zone inhibition

Activity of various Essential oils (EO) and their components on different pathogens associated with mastitis as observed by different workers is summarized in Table 2.

On perusal, it can be observed that, the most promising essential oils in terms of MIC are *Lippia salvifolia* (*S. epidermidis*, *E. coli*, *Pseudomonas* spp, *Candida albicans*), *Lippia siodoides (S. epidermidis, E.coli, Candida albicans), Pogostemom cablin ((S. aureus, S. epidermidis, S. xylosus), Juniperus virginiana (S. aureus, S. epidermidis), Leptospermum scoparium (S. aureus, S. epidermidis), Origanum vulgare (Staphylococcus spp, E.coli), Thymus vulgaris (S. epidermidis, Prototheca zopfii), Cinnamomum cassia (S. xylosus), Cinnamomum zeylanicum (Prototheca zopfii), Citrus bergamia (Prototheca zopfii) and Melaleuca alternifolia (Prototheca zopfii)*. In contrast, MIC for essential oils has been on higher side against Streptococci.

Amongst various components, only Carvacrol, Citral, Thymol and trans-cinnamaldehyde have been effective with lower MIC against all pathogens of mastitis origin. A composition comprising of terpinen-4-ol (in 1-10% conc., preferably 1-2%) as active compound in combination with one or more excipients and/or adjiuvants has been applied for protection through patent application by Paolo, 2008 intended for use in prevention and treatment of mastitis.

Broth dilution testing performed using autoclaved whole milk instead of broth to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oils alone and in combination have been used by Noori *et al*., 2019 to examine the antimicrobial effect of *Lavandula stoechas* (lavender) and *Origanum majorana* (marjoram) essential oil against mastitis-causing pathogens. MIC values ranged from 3.12-4.37% v/v and MBC between 6.25-8.75% v/v for the lavender. The MIC and MBC of the marjoram ranged from 0.62-1.87% v/v and 1.25-3.75% v/v, respectively. The MIC ranged from 2.5 - 5% v/v and MBC between 5 - 10% v/v for lavender + marjoram combination.

II. Time kill Assay and Bacterial Lysis:

Cerioli *et al*., 2018 analysed activity of *Minthostachys verticillata* EO and limonene on *E. coli*, *Bacillus pumilus* and *Enterococcus faecium* by time kill assay and bacterial lysis at concentration corresponding to MIC of the EO and limonene at a concentration that showed the best inhibitory effect. There was significant reduction in population after 6 h of treatment in case of EO, whereas, limonene showed no effect. For bacterial lysis studies, EO was able to produce 50% of bacterial lysis after 30 min. On the other hand, limonene did not produce bacterial lysis.

Time-kill assay of lavender and marjoram oils were determined in milk up to 24 h by Noori *et al*., 2019. The presence of lavender and marjoram oils at a sub-MIC concentration significantly reduced the bacterial

Table 2

Minimum inhibitory, minimum bactericidal concentrations and zone of inhibitions of different essential oils/ components as reported by different studies

Source of isolate (C/SC)*	Essential oil/content	Minimum inhibitory concentration	Minimum bactericidal concentration	Zone inhibition (mm)	Reference
***Staphylococcus* spp**					
Bovine (C) (27)	*Origanum vulgare*, Carvacrol, Thymol	0.16->10 µl/ml			Norby and Halbert (2016)
	Trans-cinnamaldehyde	0.04-0.63 µl/ml			
Coagulase Positive Staphylococci					
Cattle/ Goat (C)	*Cinnamomum zeylanicum*	C:0.8-3.2; G: 1.6-3.2 mg/ml	1.6-6.4 mg/ml		Dal Pozzo *et al.*, 2012
	Trans-cinnamaldehyde	C:0.4-0.8; G: 0.8-1.6 mg/ml	C:0.4-6.4; G: 0.8-6.4 mg/ml		
Bovine Bovine	Thymol	0.2-0.8 mg/ml	0.4-6.4 mg/ml		Dal Pozzo *et al.*, 2011
	Carvacrol	0.2-1.6 mg/ml	0.2-3.2 mg/ml		
	Lippia graveolans, *Origanum vulgare*	0.8-3.2 mg/ml	1.6-6.4 mg/ml		
	Thymus vulgaris	0.8-3.2 mg/ml	1.6-3.2 mg/ml		
	Ocimum basilicum, *Rosmarinus officinalis, Salvia officinalis, Zingiber officinale,* Cineole	R	R		
S. aureus					
Cattle (SC)	*Alpinia zerumbet*	6.3-25.00 mg/ml	-		Castro *et al.*, 2016
Sheep and Goat (C&SC)	*Allium cepa, Nigella sativa*	3.25 mg/ml	-	23±0.23, 26±0.25 (20µl)	Abdalhamed *et al.*, 2018
Sheep (SC)	*Salvia officinalis* (sage)	60 mg/ml	120 mg/ml	-	Alekish *et al.*, 2017
Bovines (C)	Carvacrol, Eugenol, Thymol, trans-cinnama-ldehyde	5, 6, 6, 1 mg/ml	12, 14, 12, 4.5 mg/ml	-	Baskaran *et al.*, 2009
Bovines (C)	*Cymbopogon citrates*	0.5-1mg/ml	-	1.4-8.5 (1%), 8-14 (5%), 17-29 (10%)	Choi *et al.*, 2012
	Origanum vulgare	1-4mg/ml	-	1-2 (1%), 2.5-5.5 (5%), 5.5-11 (10%)	
ATCC 29740	Citral, Linalool, Decanal, Valencene	0.2, 1.2, >25, >25 mg/ml			Federman *et al.*, 2016
Cow (C)	*Thymus satureoides*+ *Rosmarinus verbenone*	0.93 mg/ml			Lefevre *et al.*, 2008
Bovine (C) (18)	*Leptospermum scoparium*, *Thymus vulgaris*	0.05-6.25 µl/ml	0.78-12.5, 0.1-6.25 µl/ml		Piotr *et al.,* 2018
	Pogostemom cablin	0.1-3.13 µl/ml	0.1-3.13 µl/ml		
	Juniperus virginiana	0.1-6.25 µl/ml	0.1-6.25 µl/ml		
	Pelargonium graveolens	0.78-12.5 µl/ml	1.56-25 µl/ml		
	Cinnamomum cassia	1.56-12.5 µl/ml	1.56-12.5 µl/ml	-	
Sheep (22)	*Calamintha nepeta*	0.5-4.0 mg/ml	-	R-19.3 (5µl)	Queiroga *et al.*, 2018
	Lavandula stoechas	0.5->4.0 mg/ml	-	14.2-25.0 (5µl)	
	Rosmarinus officinalis	0.5-4.0 mg/ml	-	7.5-10.0 (5µl)	
	Thymus mastichina	0.5->4.0 mg/ml	-	9.0-11.8 (5µl)	
-	*Lippia sidoides, L. salvifolia, L. salvifolia* + *L. sidoides* (9:1)	0.63, 0.37, 0.37 µg/ml			Soares *et al.*, 2013
S. epidermidis					
Sheep and Goat (C&SC)	*Allium cepa, Nigella sativa*	12.5, 6.25 mg/ml	-	19±0.23, 24±0.25 (20µl)	Abdalhamed *et al.*, 2018

Bovine (C) (8)	*Cinnamomum cassia*	0.39-6.25 µl/ml	0.78-6.25 µl/ml	-	Piotr *et al.*, 2018
	Juniperus virginiana	0.2-6.25 µl/ml	0.39-25 µl/ml	-	
	Leptospermum scoparium	0.05-6.25 µl/ml	0.1-6.25 µl/ml	-	
	Pelargonium graveolens	1.56-12.5 µl/ml	6.25-12.5 µl/ml	-	
	Pogostemom cablin	0.2-3.13 µl/ml	0.2-12.5 µl/ml	-	
	Thymus vulgaris	0.2-3.13 µl/ml	0.39-6.25 µl/ml	-	
Sheep (22)	*Calamintha nepeta*	2.0-4.0 mg/ml	-	7.2-18.3 (5µl)	Queiroga *et al.*, 2018
	Lavandula stoechas	1.0->4.0 mg/ml	-	13.2-33.0 (5µl)	
	Rosmarinus officinalis	0.5->4.0 mg/ml	-	R	
	Thymus mastichina	4.0->4.0 mg/ml	-	R-13.8 (5µl)	
	Lippia salvifolia, L. sidoides, L. salvifolia +L. sidoides (9:1)	0.63, 0.37 µg/ml			Soares *et al.*, 2013
CNS					
Sheep/Goat(C/SC)	*Allium cepa, Nigella sativa*	3.25 mg/ml	-	19±0.23, 22±0.25 (20µl)	Abdalhamed *et al.*, 2018
S. chromogenes, S. warneri, S. xylosus, S. sciuri from Sheep/Goat (C/SC); 10 µl (1:10); Zone size in mm					Fratini *et al.*, 2014
C. bergamia	6.0, 5.7, 0.0, 6.0	*C. zeylanicum*	9.0, 7.0, 8.0, 6.7	*E. globules*	6.0, 11.3, 7.0, 11.0
F. vulgare	0.0, 8.0, 8.0, 0.0	*O. majorana*	0.0, 7.0, 6.7, 0.0	*O. vulgare* (Ov)	9.3, 10.0, 9.7, 10.0
R. officinalis	0.0, 0.0, 8.0, 0.0	*S. Montana* (Sm)	11.3, 12.0, 12.7, 11.7	*T. vulgaris ct.* carvacrol (Tvt)	8.79, 0.0, 7.0, 0.0
T. vulgaris ct. thymol	12.3, 14.7, 12.0, 10.5	ST (Sm+Tvt) (20 µl)	23.7, 20.0, 18.0, 16.3	SO (Sm+Ov) (20 µl)	12.3, 9.3, 8.6, 12.3
AB (0.52 thymol+0.47 µl carvacrol) (in 20 µl)	18.3, 11.0, 9.7, 8.0	CD (0.52 thymol+ 0.47 carvacrol + 0.26 µl p-cyeme) (in 20 µl)	21.6, 23.6, 20.0, 31.3		
S. xylosus from Bovine (C) (5)					Piotr *et al.*, 2018
EO	**MIC**	**MBC**	**EO**	**MIC**	**MBC**
Cinnamomum cassia	0.2-6.25 µl/ml	0.39-6.25 µl/ml	*Juniperus virginiana*	0.1-3.13 µl/ml	0.39-3.13 µl/ml
Leptospermum scoparium	0.78-3.13 µl/ml	0.78-1.56 µl/ml	*Pelargonium graveolens*	1.56-6.25 µl/ml	3.13-6.25 µl/ml
Pogostemom cablin	0.1-1.56 µl/ml	0.1-1.56 µl/ml	*Thymus vulgaris*	0.1-3.13 µl/ml	0.1-3.13 µl/ml
***Streptococcus* spp**					
Bovine (C) (28)	*Origanum vulgare*	5->10 µl/ml			Norby and Halbert (2016)
	Carvacrol	0.16-0.63 µl/ml			
	Thymol	0.31-0.63 µl/ml			
	Trans-cinnamaldehyde	0.16-0.63 µl/ml			
S. agalactiae					
Bovines (C)	trans-cinnamaldehyde	0.5 mg/ml	4.0 mg/ml	-	Baskaran *et al.,* 2009
	Eugenol, Carvacrol, Thymol		4 mg/ml	8, 8, 9 mg/ml	-
Cow (C)	*Thymus satureoides+ Rosmarinus verbenone*		1.88mg/ml	-	Lefevre *et al.*, 2008
S. dysgalactiae					
Bovines (C)	trans-cinnamaldehyde	0.5 mg/ml	4.0 mg/ml	-	Baskaran *et al.*, 2009
	Eugenol, Carvacrol, Thymol	4 mg/ml	4,8 ,9 mg/ml	-	
Cow (C)	*Thymus satureoides+ Rosmarinus verbenone*	0.93mg/ml			Lefevre *et al.,* 2008
S. uberis					
Bovines (C)	trans-cinnamaldehyde	1 mg/ml	4.5 mg/ml	-	Baskaran *et al.*, 2009
	Eugenol, Carvacrol, Thymol	4, 8, 6 mg/ml	5, 12, 14 mg/ml	-	
Cow (C)	*Thymus satureoides+ Rosmarinus verbenone*	0.93mg/ml	-	-	Lefevre *et al.,* 2008
Cow	*Minthostachys verticillata*	14.3-114.5mg/ml	114.5-229mg/ml		Montironi *et al.,* 2016

	Limonene	3.3-52.5mg/ml	210mg/ml		
Escherichia coli					
Sheep and Goat (C&SC)	*Allium cepa, Nigella sativa*	12.5, 6.25 mg/ml	-	19±0.23, 26±0.25 (20µl)	Abdalhamed *et al.,* 2018
Bovines (C)	Carvacrol, Eugenol, Thymol	8 mg/ml	12, 14, 15 mg/ml	-	Baskaran *et al.*, 2009
-	Carvacrol, Thymol	0.63->10 µl/ml	-	-	Norby and Halbert (2016)
Cow (SC)	Limonene	-			Cerioli *et al.*, 2018
	L. salvifolia+L. sidoides (9:1)	0.37 µg/ml			Soares *et al.*, 2013
	Lippia salvifolia	0.63 µg/ml			
	Lippia sidoides	1.25 µg/ml			
Cow (SC)	*Minthostachys verticillata*	0.9-14.51 mg/ml			Cerioli *et al.*, 2018
Bovine (C) (33)	*Origanum vulgare*	0.13->10 µl/ml			Norby and Halbert (2016)
Cow (C)	*Thymus satureoides+ Rosmarinus verbenone*	1.88mg/ml			Lefevre *et al.*, 2008
Bovines (C)	trans-cinnamaldehyde	1 mg/ml	4.5 mg/ml	-	Baskaran *et al.*, 2009
-	Trans-cinnamaldehyde	0.31-0.63 µl/ml			Norby and Halbert (2016)
Klebsiella pneumonia					
Sheep and Goat (C&SC)	*Allium cepa, Nigella sativa*	12.5, 6.25 mg/ml	-	19±0.23, 23±0.25 (20µl)	Abdalhamed *et al.,* 2018
***Pseudominas* spp**					
Sheep and Goat (C&SC)	*Allium cepa, Nigella sativa*	12.5, 6.25 mg/ml	-	13±0.56, 15±0.75 (20µl)	Abdalhamed *et al.*, 2018
-	*Lippia sidoides, L. salvifolia, L. salvifolia+ L. sidoides* (9:1)	0.37µg/ml			Soares *et al.*, 2013
Bacillus pumilus					
Cow (sc)	*Minthostachys verticillata*	1.8-29.0 mg/ml			Cerioli *et al.*, 2018
	Limonene	6.6-10.5 mg/ml			
Enterococcus faecium					
Cow (sc)	*Minthostachys verticillata*	3.63 mg/ml			Cerioli *et al.*, 2018
	Limonene	R			
Prototheca zopfii					
Cow (C)	*Mantha piperita* (peppermint)			28±1.93(50µl) more effective than Itraconazole	Cuc *et al.*, 2012
Cow (-)	*Mantha piperita*	R			Grzesiak *et al.,* 2018
Cow (C)	*Melaleuca alternifolia* (tea tree)			30±1.63(50µl) more effective than Itraconazole	Cuc *et al.,* 2012
Cow (C)	*Melaleuca alternifolia*	0.03-0.12 µg/ml			Tortorano *et al.*, 2008
Cow (-)	*Thymus vulgaris*	0.6-1.0 µl/ml			Grzesiak *et al.*, 2016
Cow (-)	*Thymus vulgaris*	0.25-1.0 µl/ml			Grzesiak *et al.*, 2018
Cow (C)	*Abies alba*(fir), *Vitis vinifera* (Grape seed), *Oreganum compactum* (oregano)			R	Cuc *et al.*, 2012
Cow (-)	*Satureja hortensis* (savory)			21±3.29(50µl) comparable to Itraconazole	Cuc *et al.*, 2012
Cow (-)	*Cinnamomum zeylanicum*	0.2-0.4 µl/ml			Grzesiak *et al.*, 2016
	Lavandula angustifolia	6.0-8.5 µl/ml			
	Ocimum basilicum	4.5-5.5 µl/ml			
	Pelargonium graveolens	3.5-4.0 µl/ml			
	Rosmarinus officinalis	8.5-10.5 µl/ml			

	Salvia sclarea	1.5-2.5 µl/ml	
	Syzygium aromaticum	0.8-1.0 µl/ml	
Cow (-)	*Allium ursinum*	R	Grzesiak *et al.*, 2018
	Origanum majerana	0.25-0.5 µl/ml	
	Origanum vulgare	0.5-1.0 µl/ml	
Cow (C)	*Citrus bergamia*	0.15-5 µg/ml	Tortorano *et al.*, 2008
Candida albicans			
Bovine (C) (MIC80)	*Origanum floribundum*	17.18-23.14µg/ml	Ksouri *et al.*, 2017
	Rosmarinus officinalis	23.99-31.08µg/ml	
	Thymus ciliates	15.02-20.96µg/ml	
-	*Lippia salvifolia, L. sidoides*	2.50µg/ml	Soares *et al.*, 2013
	L. salvifolia + L. sidoides (9:1)	1.25 µg/ml	

* C- clinical, SC - subclinical

population in 4, 10 and 24 h. Generally, essential oil of marjoram had greater antibacterial activity than lavender against all mastitis-causing pathogens tested.

Baskaran *et al.*, 2009 used a concentration greater than the MBC of Trans-cinnamaldehyde (TC) for Time-Kill assay in milk. In all the treatment samples, bacterial counts reduced substantially, reducing bacterial population by 4.0 to 5.0 log10 cfu/mL and to undetectable levels in 6 and 24 h, respectively. Also, for all bacteria, TC at MIC concentration did not allow growth to occur. They also determined persistence of antimicrobial activity of TC in milk by inoculating the milk samples with *S. aureus* every 48 h until day 6. Bacterial reduction was observed on the subsequent days, *S. aureus* inoculated on day 6 was decreased to undetectable levels on day 12 of the experiment (more reduction at 0.7% as compared to 0.45% TC).

Time-dependent inhibitory effects of citral and linalool on *S. aureus* growth have been assessed by Federman *et al.*, 2016. Broth supplemented with citral (0.02 or 0.04 %) or linalool (0.12 or 0.24 %) were used to grow *S. aureus*. Concentrations of 0.04% citral and 0.24% linalool inhibited the growth of *S. aureus* below detectable limits after 24 h of treatment. This inhibitory effect was maintained for 72 h for both citral and linalool; however, the inhibitory effect was reduced after 48 h for both citral and linalool.

III. Effect on Biofilm formation and on Mature Biofilm:

Previously determined MIC levels of *Minthostachys verticillata* EO and limonene at its best inhibitory effect added to a bacterial suspension (*B. pumilus*) in polystyrene plate decreased biofilm, which was not statistically significant in respect to control. Inhibition percentages from 36.51 to 89.60% with EO and from 22.06 to 89.83% with limonene were observed during biofilm formation and inhibition percentages of EO from 35.06 to 66.35%, and limonene from 33.36 to 61% were observed on mature biofilm (Cerioli *et al.*, 2018).

Syzygium aromaticum EO (EOSA) and *Cinnamomum zeylanicum* EO (EOCZ), eugenol and cinnamaldehyde at final concentration of 0.106 mg/mL in tryptone soy broth were tested for their effect on production of biofilm by *S. aureus* on polystyrene and stainless steel. Significant differences in biofilm production were noted between isolates grown in the absence and presence of EO and major components. While, a statistically significant reduction in biofilm formation was observed in presence of both EOs and cinnamaldehyde on both polystyrene and stainless steel, eugenol reduced biofilm formation only on polystyrene (Budri *et al.*, 2015).

Preformed biofilms generated by *S. aureus* have been treated with unsupplemented nutrient broth or nutrient broth supplemented with either citral (0.02, 0.04 or 0.08 %) or linalool (0.12, 0.24 or 0.48 %) by Federman *et al.*, 2016. All concentrations tested, which included the MIC values above, were capable of inhibiting pre-formed biofilms. The most effective concentrations were 0.08% citral, 0.24% linalool and 0.48% linalool.

Antibacterial activity of limonene has also been tested against produced biofilms produced by *S. uberis*. Montironi *et al.*, 2016 tested limonene and *Minthostachys verticillata* EO effect on preformed biofilm at their respective MICs. Both of them were able to reduce biofilm production. The percentages of inhibition of EO MIC were from 88.25 ± 7.62 to 23.50 ± 16.26 and of limonene were from 92.18 ± 4.78 to 23.20 ± 16.05.

IN VIVO STUDIES

Although there are many studies to assess the effect of essential oils and their components on various isolates of mastitic origin *in vitro*, there are only a few *in vivo* studies to evaluate their therapeutic efficacy.

Lefevre *et al.*, 2008 evaluated the therapeutic efficiency of the intramammary infusion of three essential oils (*Thymus vulgaris, Rosmarinus verbenone* and *Laurus nobilis*). Fifty-five cases of mastitis were treated (Intramammary infusion) with 10 ml of a mixture of the three oils (1.5% each in sunflower oil). Forty-five others were treated with 10 ml of a mixture of *Thymus vulgaris* and *Rosmarinus verbenone* (6% of each in sunflower oil or in water). The recovery rate was only 40%, which was found to be unsatisfactory. However, the preparation showed very good tolerance, and no local or general reactions were found.

Mastilep gel and Herbal spray (AV/AMS/15) comprising of *Cedrus deodara, Curcuma longa, Eucalyptus globules, Glycyrrhiza glabra* have been evaluated by Hase *et al.*, 2013 for therapeutic effect on subclinical mastitis in bovines. The preparations were applied two times a day post milking for 5 days individually. Herbal spray (AV/AMS/15) and Mastilep gel achieved 60% cure rate, there was reduction in somatic cell count, improvement in milk yield and no adverse effect on milk fat content was observed.

Montironi *et al.*, 2019 characterized the effect of *M. verticillata* EO on macrophage phagocytosis and evaluate its immunomodulatory and protective effects in murine model of *Enterococcus faecium* mastitis. It was found that EO activated macrophage phagocytosis mechanisms resulting in generation of reactive oxygen species. Also, EO modulated innate immune response in mammary glands decreasing the infiltration of polymorphonuclear neutrophils and IL-1β and TNF-α mRNA expression. EO increased the expression of IL-10 in the last hours of infection. However, EO did not increase the number of activated CD4+ or CD8+ T cells or the production of specific antibodies.

In another study by Cho *et al.*, 2015, Oregano EO ointment containing 0.9 mL of EO in 10 ml tube was infused into the inflamed quarters twice a day for 3 days in single and double doses. There was improvement in udder conditions. In both the groups, SCCs decreased significantly compared with those before treatment. Also *S. aureus* and *E. coli* were not detected in the milk on the fourth day after the treatment for 3 days. Similar efficacy was observed for WBC numbers which decreased significantly compared with those before the treatment.

Similar study has been carried out to observe the therapeutic efficacy of intramammary infusion of Sage (*Salvia officinalis*) (5 ml solution with 0.2 ml DMSO, 1 ml EO and 3.8 ml sterile saline) into each teat of ewes suffering from subclinical mastitis twice per day for 3 consecutive days (Alekish *et al.*, 2017). SCC decreased significantly at after 24 and 48 hrs. Milk fat and lactose increased significantly, while no significant changes were observed in percentages of solids-not-fat, protein and total solids. Also, no changes were observed for any of the hematology or serum biochemical parameters. There were no local or systemic side effects observed in any of the treated ewes.

DISCUSSION

There are many *in vitro* studies for evaluating the efficacy of essential oils for their antimicrobial action against many isolates of mastitic origin and many essential oils and their components have been found to be effective with a wide range of MIC and MBC. Contrarily, there are only a few *in vivo* studies to assess their therapeutic effects. Some patent applications have been filed claiming pharmaceutical efficacy of various essential oils. E.g, Soares *et al.*, 2013 have formulated a compostion comprising essential oils of *Lippia salvifolia* and *Lippia sidoides* within the range between 9:1 and 1:9, so to supply at least 50% thymol and 5% carvacrol in the form of gel, cream, ointment and solution.

Patent application by Rajamannan, 1993 is for an infusible product for use in the treatment of bovine mastitis comprising primarily of diacetyl and a trace amount of acetoin +Tea tree oil (Maleluca). Tea tree oil has also been utilized for formulating a phyto-derivative solution for endo-mammary anti-mastitis treatment. *Melaleuca alternifolia* EO having a minimum terpinen-4-ol content of 35% and a maximum 1,8 cineole content of 5%; (Manzoni, 2002).

One commercial product known as Phyto-Mast comprises of essential oils of *Angelica sinensis*, *Gaultheria procumbens*, *Glycyrrhiza uralensis* and *Thymus vulgaris*. Mullen, 2020 in his report found that only Thymus vulgaris oil had consistent antibacterial activity against all three pathogens tested and combination of oils did not show typical dose-response effects. He also suggested that limited antibacterial activity of Phyto-Mast *in vitro* prompts further study on the action of essential oils in the mammary gland.

However, may seemingly be effective *in vitro*, issues have been raised about their use as therapeutic agents. Norby and Halbert (2016) have suggested that although MICs are low for essential oils, as they get diluted in the milk in the quarter, they need to be applied at high concentration. Such high concentration will be too irritating and may cause damage to mammary tissues.

Venkitanarayanan, 2013 in their report on project related to investigation of potential of natural antimicrobials for controlling bovine mastitis have reported increased signs of discomfort like tail flicking and kicking in dose response manner when transcinnamaldehyde was infused in mammary gland. Also, SCCs increased 100-fold.

CONCLUSIONS

Further clinical trials are warranted to determine safety and possible withdrawal times in milk before its recommendation for use in organic operations. Owing to their wonderful antimicrobial activity, if their tissue irritant properties can be mitigated by some manipulations, they may be of immense help in fighting mastitis in future.

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