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ANTIBACTERIAL PROPERTIES OF *Ocimum sanctum, Moringa oleifera* AND *Murraya koenigii* LEAF EXTRACTS AGAINST *Corynebacterium pseudotuberculosis* ISOLATED FROM CAMEL (*Camelus dromedarius*)

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ABSTRACT

In the present study, antibacterial properties of methanolic and chloroform extracts of *Ocimum sanctum*, *Moringa oleifera* and *Murraya koenigii* leaves against *Corynebacterium pseudotuberculosis* (CPs) isolated from abscess in dromedary camel were screened using agar well diffusion assay and minimum bactericidal concentration (MBC) were determined using broth microdilution technique. MBC of selected plant extracts varied from 3.125 to 12.5 mg/ml. Chloroform extracts of *Ocimum sanctum* and *Murraya koenigii* had highest antibacterial properties; while it was lowest in methanolic extract of *Moringa oleifera* and *Murraya koenigii* leaves.

Key words: Antimicrobial, Camel, Corynebacterium, Moringa, Murraya, Ocimum

Corynebacterium pseudotuberculosis (CPs) is an important pathogen of domestic animals including sheep, goat, horses, cattle and camel (Dorella *et al*, 2006). In camel, it causes enlargement and suppuration of peripheral and visceral lymph nodes (Tejedor-Junco *et al,* 2004; Wernery and Kinne, 2016; Ranjan *et al,* 2018) and plays an important role in superficial septic wounds (Zidan *et al,* 2013). *In vitro* sensitivity tests suggest that CPs isolated from different animal species are sensitive for most of the common antibiotics (Judson and Songer, 1991). However, after growing CPs as biofilm, in an attempt to mimic the environment of natural infection, the bacterium was found highly resistant to all the tested antimicrobials (Olson *et al,* 2002). Research reports published in the recent past also suggest emergence of drug resistance problem in CPs (Abdel-Wahab and Shigidi, 2013; Algammal, 2016).

There are many medicinal plants containing useful phytochemical constituents which have antibacterial properties and their therapeutic values help in treating many bacterial infections (Abuga *et al*, 2021). Masese *et al* (2016) found *Moringa oleifera, Murraya koingii and Ocimum sanctumare* as an effective reducing agent for the synthesis of AgNPs which were highly stable and had significant activity against *Escherichia coli* and *Staphylococcus aureus*. Moodley *et al* (2018) found that the biosynthesised nanoparticle preparations from *M. oleifera* leaf extracts exhibit potential for application as broad-spectrum antimicrobial agents. The extract of leaves of *Ocimum sanctum* has demonstrated effective antimicrobial property against *A. actinomycetemcomitans*, suggesting its possible use as an effective and affordable "adjunct" in the management of periodontal conditions in humans (Mallikarjun *et al*, 2016). *Murraya koenigii* extracts have demonstrated antibacterial effects particularly on *E. coli* and *Staphylococcus* as compared to antibiotics such as Gentamycin and Amikacin (Irfan *et al,* 2016). Fouad (2019) found antibacterial efficacy of *Moringa oleifera* leaf extract against pyogenic bacteria isolated from a dromedary camel (*Camelus dromedarius*) abscess. Zubair (2020) evaluated the inhibitory effect and anti-biofilm activity of *Moringa oleifera* and *Citrus sinensis* extracts against those of pathogenic *Pseudomonas*

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aeruginosa and *Staphylococcus aureus* and found that extracts have effectively blocked MRSA and ESBL development in the biofilm matrix. However, studies on antibacterial properties of medicinal plant extracts in camels is meager. The present investigation is, therefore, undertaken to study the antibacterial properties of *Ocimum sanctum, Moringa oleifera* and *Murraya koenigii* leaf extracts against *Corynebacterium pseudotuberculosis* isolated from an abscess of a camel (*Camelus dromedarius*).

Materials and Methods

Bacterial culture and characterisation

The pure colonies of CPs were isolated from pus sample from enlarged, suppurated, cervical lymph node of a dromedary camel housed at ICAR-National Research Centre on Camel, Bikaner, India. The organism was identified on the basis of culture characteristics, morphological features after Grams staining, result of various biochemical tests (Nabi, 2021) and quadruplex PCR test (Almeida *et al,* 2017).

Collection of plants, identification and extract preparation

Fresh leaves of *Ocimum sanctum*, *Moringa oleifera* and *Murraya koenigii* were collected from different localities of Bikaner, Rajasthan and were authenticated by scientists from ICAR-Central Institute for Arid Horticulture, Bikaner. Collected leaves were rinsed with distilled water, dried in shed at room temperature for 15 days and ground to obtain a coarse powder. Methanolic and chloroform extracts of each plant leaves were prepared. Fifty gram dried leaf powder was soaked overnight in 200 ml methanol/ chloroform and the mixture was shaken vigorously several times in between. Next day, the mixture was filtered with Whatman filter paper number one. The filtrate was evaporated to dryness *in vaccuo* at 50°C using rotary film evaporator, and stored at -20°C till further use. Extractability or yield (%) of each extract was calculated using a formula: Extractability or yield (%) = (weight of extract obtained in gram/ weight of leaf powder taken in gram) *100 (Mazhangara *et al,* 2020).

The extract obtained was dissolved in 10% dimethylsulfoxide (DMSO; HiMedia Laboratories Private Limited, Mumbai, India) to a desired concentration and sterilised by passing through a 22 µm (pore size) filter (HiMedia Laboratories Pvt Ltd., Mumbai, India) before use.

Evaluation of anti-microbial properties of plant extracts

The antimicrobial activities of plant-extracts were screened using agar well diffusion test (Kavitha, 2017) with slight modifications. Briefly, 0.2 ml of diluted inoculum ($1X10^5$ CFU/ml) of the CPs was swabbed on the Brain Heart Infusion (BHI) Agar supplemented with 5% defibrinated sheep blood. Thereafter, using a sterilised cork borer, wells of 5 mm diameter were punched. Using a micropipette, 100 µl of the plant extract solution (100 mg/ml) were added to the wells. The plates were incubated aerobically at 37±2 °C for 24 to 48 h and the zone of inhibition (in mm) was measured with the help of a Vernier caliper. The test was performed in triplicates with controls.

The minimum bactericidal concentrations (MBC) of the methanolic and chloroform extracts were determined using broth micro-dilution technique as per the standard CLSI methods (Wayne, 2008) with slight modifications (Jahan *et al,* 2011).

Standardisation of inoculum size

To ensure an exact number of bacteria present in the inoculum, one or two isolated colonies were inoculated into 5 ml of BHI broth (supplemented with 0.1% Tween 20 to prevent clumping of colonies) and inoculated at 37°C for 24-48 hours. Thereafter, the inoculum was centrifuged at 4,000 rpm for 5 minutes with appropriate aseptic precautions. The supernatant was discarded and the pellet was re-suspended in sterile Phosphate Buffer Saline (PBS) pH 7.4 and centrifuged again at 4000 rpm for 5 min. The process was repeated until the supernatant was clear. The pellet, thereafter was suspended in 5 ml sterile PBS and optical density was recorded at 500 nm. Serial dilutions (two-fold) were done in PBS under aseptic conditions until the optical density was in a range of 0.8 to 1.0. The actual number of colony forming units was estimated after inoculating 100 µl of the bacterial suspension over BHI Agar with 5% defibrinated sheep blood and incubating at 37°C for 24-48 hours. The required dilution factor was calculated and the dilution was carried out to obtain a final concentration of $5 X 10^6$ cfu/ ml (Norman *et al,* 2014).

Preparation of the microplate

In a sterile 96 micro well plate, different test herbal solutions (200 µl each) were pipetted into the first row under aseptic conditions. To all other wells, 100 µl of BHI broth was added. Serial dilutions were performed by transferring 100 µl from the corresponding wells in the first row to the next row

so that at the end, 100 µl of the test herbal extract was present in each well in a serially descending concentration (50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.391, 0.195 and 0.098 mg/ml). Now, 60 µl of 3.3 X strength BHI broth, 20 µl sterile PBS and 20 µl of bacterial suspension (5 X 10^6 cfu/ ml) was poured into each well to obtain a concentration of 5 X 10^5 cfu/ ml and a final volume 200 µl in each well. In addition, one media control (herbal extract and bacterial culture), one bacterial control (herbal extract and BHI broth) and one extract control (BHI broth plus bacterial culture) were also run simultaneously. The micro well plate was covered with lid and sealed with tape and incubated at 37°C for 48 hours. The MBC value was determined by sub-culturing each test dilutions (by transferring 100 µl) on BHI agar with 5% defibrinated sheep blood plates at 37°C for 24-48 hours. The highest dilution (or lowest extract concentration) showing no bacterial growth was taken as MBC (Norman *et al*, 2014).

Statistical analysis

The values obtained were analysed by one-way ANOVA using computer software Statistical Package of Social Sciences (SPSS-20).

Results and Discussion

Plant extracts

The colour, consistency and extractability percentage of different plant extracts are given in table 1. The methanolic extract of *Ocimum sanctum* appeared as blackish, semi-solid paste like with extractability 5.58%. The chloroform extract was brown-black coloured flakes with extraction per cent 2.80. In corroboration with the present findings Agarwal *et al* (2010) reported extractability of ethanolic *O. sanctum* leaf extract to be 6%. Likewise, Shafi *et al* (2018) reported the extraction percentage for hydro-alcoholic extract (1:1 water and ethanol) to be 9.6%.

Methanolic *M. oleifera* leaf extract obtained was brown-blackish, semi solid mass with 7.32 % yield. The chloroform extract was dark green in colour with extractability 2.32%. Nikkon *et al* (2003) reported extraction percentage of *M. oleifera* root barks to be 3.33 % using ethanol as extraction media.

Methanolic leaf extract of *Murraya koenigii* was brown-black solid mass with 0.81 % extractability. The chloroform extract was also similar in colour and consistency with extractability was 1.70%. Vats *et al* (2011) reported extraction percentage of *M. koenigii* roots as 4.03, 1.31, 0.59 and 9.4% for petroleum ether, chloroform, ethyl acetate and ethanol extracts, respectively. Kavitha (2017) reported that organic extracts obtained were viscous in nature and brownish in colour, but yield percentage recorded was 7.0, 6.5 and 9.2 % (v/w) for hexane, chloroform and ethanol, respectively.

Evaluation of antibacterial property

Results of agar well diffusion test and estimation of Minimum Bactericidal Concentration

Table 1. Colour, consistency and extractability percentage of different plant extracts used with methanol and chloroform as solvents.

S. No.	Plant	Solvent used	Colour	Consistency	Extractability %
1.	*Ocimum sanctm*	Methanol	Blackish	Semi-solid (Paste like)	5.58
		Chloroform	Brown-black	Powder (Flakes)	2.80
2.	*Moringa oleifera*	Methanol	Brown-black	Semi-solid	7.32
		Chloroform	Dark green	Semi-solid	2.32
3.	*Murraya koenigii*	Methanol	Brown-black	Semi-solid	0.81
		Chloroform	Brown-black	Semi-solid	1.70

Table 2. Zone of inhibition and MBC of different plant extracts against *C. pseudotuberculosis*.

S.No	Plant	Solvent used	Zone of inhibition(mm)*	MBC (mg/ml)
1	*Ocimum sanctum*	Methanol	12.667 ± 0.667^{a}	6.25
		Chloroform	14.000 ± 1.000^{ab}	3.125
2	*Moringa oleifera*	Methanol	14.667 ± 0.667^{abc}	12.5
		Chloroform	16.00 ± 1.555^{bc}	6.25
3	*Murraya koenigii*	Methanol	14.667 ± 0.667^{abc}	12.5
		Chloroform	17.000 ± 0.577^{c}	3.125

*Data are presented as mean ±S.E. as measurement of inhibition zone (mm). Means and standard errors determined from 3 biological replications. The values with different superscript differ significantly ($P< 0.05$) within a column.

(MBC) are given in table 2. In the wake of emerging problem of multidrug resistant bacteria there is a continuing need for new preparations particularly low cost natural products as they are readily accepted by patients (Martin and Ernst, 2013). Nevertheless, antibacterial properties of a plant extract in *in vitro* tests is reported to vary with several factors like type and strain of bacteria, inoculum size, type of media, type of solvent and extraction procedure, part of the plant used and time of collection and total amount of herbal extract used (Mandal *et al,* 2012).

Osmium sanctum

The zone of inhibition for methanolic and chloroform extracts of *O. sanctum* varied from 12 to 15 mm in diameter. In corroboration of the present findings, Aqil *et al* (2005) reported that inhibition zone for ethanolic extract of *O. sanctum* leaves against methicillin resistant *Staph. aureus* (MRSA) varied from 11 to 18 mm in diameter when 100 µl of plant extract with concentration 100 mg/ml was used. An inhibition zone of 10 mm diameter was reported when aqueous or ethanolic extracts of *O. sanctum* were used in concentration 10 mg/ml against *Aeromonas hydrophila* (Harikrishan and Balasundaram, 2008). Agarwal *et al* (2010) reported that inhibition zone of ethanolic extract (at concentration 10 mg/ml) of *O. sanctum* varies from 12 to 19 mm in diameter against *Strepto. mutans*. Jahan *et al* (2011) also recorded zone of inhibition of ethanolic extract of *O. sanctum* against *Staph. aureus* to vary between 10.66 to 15.66 mm.

MBC for methanolic and chloroform extract of *O. sanctum* were found to be 6.25 and 3.125 mg/ml, respectively. MIC of ethanolic extract against different strains of MRSA turned to vary from 1.3 to 8.2 mg/ml (Aqil *et al,* 2005). Likewise, initial MIC of ethanolic extract of *O. sanctum* leaves ranged from 2.4- 9.4 mg/ml and 4.7- 18.8 mg/ml for resistant and sensitive *Staph. aureus* strains, respectively (Jahan *et al,* 2011). Goyal and Kaushik (2011) reported that MIC of ethanolic and methanolic extracts of *O. sanctum* leaves varied from 1024 to > 4096 µg/ml. In the present study, chloroform extract of *O. sanctum* had more potent antibacterial activity than methanolic extract. In concurrence with this observation, Shokeen *et al* (2005) also recorded that chloroform extract of leaves of *O. sanctum* have highest percentage of inhibition of *Neisseria gonorrhoeae* among hexane, benzene, chloroform, ethyl acetate, acetone and 70% ethanol extracts. However, perusal of available reports suggests large variation in MIC and MBC values of *O. sanctum* leaf extracts against different bacteria. MIC as low as 0.25 mg/ml was reported by Adiguzel *et al* (2005) and 0.02 mg/ml by Akinvemi *et al* (2005). On the other hand, Shafi *et al* (2018) reported MIC to vary from 62.5 to 125 mg/ml for hydro-alcoholic extract of *O. sanctum* leaves against common mastitis pathogens like *Staph. aureus*, *Strepto.* spp, *E. coli*, *Coryne.* spp, *Pseudomonas* spp and *Klebsiella* spp. They further reported that MIC for *Coryne.* spp was 62.5 mg/ml.

O. sanctum, a plant from family Labiatae, known as *Tulsi* in hindi, have several pharmacological activities, like hypoglycemic, antipyretic, analgesic, anti-inflammatory, antistress, immune-modulatory, radio-protective, anti-tumour and anti-bacterial (Bhargava and Singh, 1981; Godhwani *et al,* 1987).

Moringa oleifera

Mean diameter of inhibition zone for methanolic and chloroform extract of *M. oleifera* recorded was 14.68 and 16.00 mm, respectively. Rahman *et al* (2009) studied antibacterial activity *M. oleifera* leaves against 4 gram negative and 6 gram positive bacteria and found that zone of inhibition for fresh leaf juice varied between 15.23 to 25.2 mm, for powder from fresh leaf juice was 29.25 to 42.3 mm and ethanol extract of fresh leaves was 16.25 to 21.5 mm. Peixoto *et al* (2011) also reported that mean diameter of zone of inhibition for aqueous and ethanolic extract of *M. oleifera* leaves varied from 14.4 to 30.0 mm depending upon the type and concentration of extract used and genus of the test bacteria. Among ethanol, chloroform and hexane extracts of *M. oleifera* leaves, methanolic extract was found most effective against *E. Coli*, *S. dysenteriae*, *Salmonella* spp., *Enterobacter* spp., *K. pneumonia*e and *S. marcescens* (Rahman *et al,* 2010). Large variation in MIC has been reported (from 0.041 to 50 mg/ml) for crude seed extracts (ethanolic or chloroform extracts) against gram negative organisms (Chandrasekhar *et al,* 2020).

MBC of methanolic and chloroform extract of *M. oleifera* was found to be 12.5 and 6.25 mg/ml, respectively. Likewise, Fouad *et al* (2019) reported that MIC values of cold water extract and ethanolic extract of *M. oleifera* leaves were 25 mg/ml and 390 µg/ ml, respectively against CPs isolated from pus in camel. However, lower MIC values were reported in several other studies. For example, the MIC values of methanolic, ethyl acetate and hexane extracts of *M. oleifera* leaves against some Gram negative bacteria were reported to vary from 62.5 to 1000 µg/mL (Rahman *et al,* 2010). Recently, Garcia-

Beltran *et al* (2020) reported significant antibacterial activity of ethanolic and aqueous extracts against pathogenic *Vibrio anguillarum* and *Photobacterium damselae* strains at a concentration ranging from 0.25 to 1.00 mg/ml. They further opined that antibacterial activity of *M. oleifera* extracts could be attributed to certain active components that might act in a synergistic way to inhibit the bacterial growth and viability. Deoxy-niazimicine extracted from *M. oleifera* leaves is reported to be effective against several pathogenic bacteria (Nikkon *et al,* 2003). Flavonoids and glucomoringin present in *M. oleifera* also attribute antibacterial potential against certain bacteria (Onsare and Arora, 2015; Galuppo *et al,* 2013). Low levels of the isothiocyanate derivative compounds are some additional constituents that inhibit some Gram-negative and Gram-positive bacteria.

Moringa or *Drumstick tree* (*Moringa oleifera*) is widely cultivated in tropical to subtropical regions across the world (García-Beltran *et al,* 2020). The dry leaves of the plant contain high concentration of macro and micronutrients, tannins, sterols, saponins, trepenoids, phenolics, alkaloids and flavanoids (Gopalkrishnan *et al,* 2016). Antibacterial activity of *M. oleifera* leaf extract could be attributed to phenolic compounds, flavonoids, saponin, tannin and cyanogenic glycosides (Rauha *et al,* 2000; Doughari *et al,* 2007; Verma *et al,* 2009; Garcia-Beltran *et al,* 2020). However, marked variation in phytochemical composition and thereby antioxidant and antimicrobial activities of 13 different cultivars of *Moringa oleifera* obtained from different locations across the globe has been reported (Ndhlala *et al,* 2014) that may result into variation in antibacterial potential of different extracts.

Murraya koenigii

Zone of inhibition for methanolic and chloroform extracts of *M. koenigii* leaves varied from 14 to 21 mm in diameter. Likewise, in a study zone of activity (inhibition zone) ranged from 6 to 20 mm in diameter depending upon the organic solvent used and species of bacteria (Naz *et al,* 2015). In this study methanolic leaf extract showed most promising antibacterial agent among n-hexane, acetone and methanolic extract. Nagappan *et al* (2011) also reported that the diameter of inhibition zone for carbazole alkaloids and essential oil of *Murraya koenigii* varies from 8.0 mm to 18.0 mm and highest susceptibility with inhibition zone of 18.5 ± 0.5 mm, 18.5 ± 0.5 mm and 18.0 ± 0.5 mm was recorded against *S. aureus*, *P. aeruginosa* and *S. pneumoniae*, respectively.

The MBC of *M. koenigii* leaf extracts was recorded as 3.125 and 12.5 mg/ml for chloroform and methanolic extracts, respectively. Present study indicated that chloroform extract of *M. koenigii* leaves has higher antimicrobial activity than methanolic extract. MIC of chloroform extract of *M. koenigii* leaves was 125µg/ ml against *Kleb. pneumoniae*, while methanolic extract had no antimicrobial activity against tested bacteria and fungi (Panghal *et al,* 2011). On the contrary, Kavitha (2017) reported that ethyl alcohol extract displayed highest antibacterial activity when compared to hexane and chloroform extracts of *M. koenigii* leaves. They recorded MIC and MBC of ethyl alcohol extract of *M. koenigii* leaves against different bacteria which varied from 12.05 to 25 mg/ml and 25 to 50 mg/ml, respectively. Likewise, Rath and Padhy (2014) observed that MIC and MBC of methanolic extract of *M. koenigii* leaves against different multidrug resistant gram positive and gram negative bacteria causing urinary tract infections in humans varied from 1.41 to 21.67 mg/ml.

Murraya koenigii (Linn) *Spreng.,* commonly known as "*Curry patta*" in Hindi, is a member of the family Rutaceae. The antimicrobial activity of *M. koenigii* leaves is largely attributed to several carbazole alkaloids present in it (Naz *et al,* 2015; Nalli *et al,* 2016). Seven pyranocarbazoles with antibacterial activities were extracted from methanolic extract of *Murraya koenigii* (L.) through bioassay guided fractionation (Joshi *et al,* 2017).

In the present study, highest antibacterial activity is recorded in chloroform extract of *Ocimum sanctum* and *Murraya koenigii* and lowest in methanolic extract of *Moringa oleifera* and *Murraya koenigii* leaves. Variations in the phytochemical levels of the different cultivars may be an important factor behind variation recorded in antibacterial activity. The antibacterial compounds from these plants may be used as a constituent of topical antiseptic preparations.

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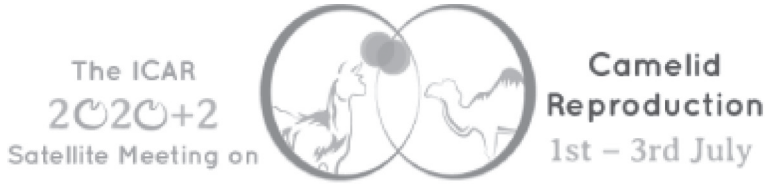
News

ICAR SATELLITE MEETING ON CAMELID REPRODUCTION



The Satellite Meeting took place from 1 (Friday) to 3 (Sunday) July 2022 following the ICAR 2020 conference at the University of Bologna, Italy. The official language of the meeting was English and no translation was provided. The scientific program includes original and review presentations on all aspects of reproduction, genetics, production and welfare in New and Old World Camelids. The major topics covered were male and female reproductive physiology and endocrinology, gynecology, theriogenology and reproductive efficiency; andrology and artificial insemination; superovulation and embryo transfer; cryo-preservation of gametes and other assisted reproductive methods; gestation, parturition and neonatology; mammary gland and milk production; camelid breeding and genetics; interaction between genotype and phenotype and camel behaviour and welfare. The papers were presented in 8 sessions, i.e. embryology, female reproduction, male reproduction (two sessions), nutrition, milking, welfare, cell culture and genetics. A field trip to Ferrara and visit of Azienda Caretti dairy and Parmigiano cheese factory also took place.

INTERNATIONAL CONFERENCE ON THE SAFETY OF CAMELS

The First International Conference on the Safety of Camels will be organised jointly by Camel Club and International Camel Organisation on 27th July 2022 at Riyadh. The opening session will be on the concept of tampering in camels followed by first session on the effect of plastic surgery on camels and session two on the effect of drugs and hormones on behaviors and appearance. The invited speakers are from USA, Saudi Arabia, Egypt, Sudan and Bahrain.

CAMEL DAIRY MARKET 2022-2027

The global camel dairy market size reached a value of US$ 6.9 Billion in 2021. Looking forward, IMARC Group expects the market to reach US$ 8.6 Billion by 2027, exhibiting a CAGR of 3.1% during 2022-2027. On a regional level, the market has been classified into Middle East, Africa, Asia, Oceania, and Rest of the world, where Africa currently dominates the global market. Some of the major players in the global camel dairy market include Camelicious, Al Ain Dairy, Desert Farms, Vital Camel Milk, Tiviski Dairy, Camilk Dairy, Camel Dairy Farm Smits, Camel Milk Co Australia and Camel Milk South Africa.