

Research Article



Prevalence of Hemorrhagic Septicemia and Use of *Moringa oleifera* and *Eucalyptus camaldulensis* Extracts against Buffalo *Pasteurella multocida* Isolates

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Abstract | A cross sectional study was conducted to estimate the prevalence rate of Hemorrhagic septicemia (HS) in buffalo at various talukas of district Shaheed Benazirabad (SBA) and to observe the efficacy of drumstick tree (*Moringa oleifera*) and river red gum (*Eucalyptus camaldulensis*) plant extracts against *Pasteurella multocida*. A total of 10 farms of each taluka of Shaheed Benazirabad district (i.e. Nawabshah, Qazi Ahmed, Daur and Sakrand) were visited to examine animals suspected of being infected with HS. A total of 2244 buffaloes and 576 buffalo calves were investigated. Among the suspected animals 100 (blood samples) were cultured, and *P. multocida* isolates were biochemically characterized. The overall prevalence rate among the observed animals was found to be 11.10% (249 cases) in buffaloes and 9.20% (53 cases) in buffalo calves in the SBA district. The highest prevalence rates were observed in buffaloes (19.59%) and buffalo calves (15.87%) in Qazi Ahmed taluka, followed by buffaloes (15.14%) in Nawabshah and buffalo calves (11.48%) in Daur. The minimum inhibition concentration (MIC) of *Moringa oleifera* and *Eucalyptus camaldulensis* was 0.10 and 0.50mg/ml, respectively against *P. multocida*. The extract of *Moringa oleifera* significantly ($P<0.05$) inhibited the *P. multocida* growth by formation of inhibitory zone diameter 18.33, 15.33, 14.00, 13.00, 10.67 and 9.33mm at concentration level of 2.50, 2.00, 1.50, 1.00, 0.50 and 0.10mg/ml, respectively in contrast to that of *Eucalyptus camaldulensis* where the zone diameter was 13.00, 9.67, 9.33, 8.33 and 8.33mm at concentration 2.50, 2.00, 1.50, 1.00 and 0.50mg/ml, respectively. The average inhibition zone formed by *Moringa oleifera* (13.55mm) was noted markedly higher ($P<0.05$) than that formed by *eucalyptus camaldulensis* (8.75mm). It could be concluded that the *Moringa oleifera* plant extract found to be efficient against *P. multocida* compared to that of *Eucalyptus camaldulensis*.

Keywords | Prevalence, Efficacy, *M. oleifera*, *E. camaldulensis*, *P. multocida*.

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Hemorrhagic septicemia (HS) especially in tropical regions like South and South-east Asia, Middle East, North-East and South Africa is one of most economically important bacterial diseases of animals (Shivachandra et al., 2002). It causes mortality in the livestock at various areas of the world where mainly buffalo and cattle are affected, hence the disease is among the fatal bacterial diseases in these animals. In Pakistan and India, the economic losses are estimated as 1.15 billion USD only from bovine death (Michael et al., 2021). The occurrence of this disease is mostly contributed due to changes in weather particularly when monsoon season starts, in addition to malnutrition as aiding disease factor. In cattle and buffalo HS disease is caused by *Pasteurella multocida*, a gram-negative bacterium of 0.2 to 0.4 × 0.6 to 2.5 μm size, non-motile, non-spore forming, coccobacillus and facultative anaerobic bacterium (De Alwis et al., 1999). The treatment of the disease because of its rapid development is often failed but antimicrobials are administered in earlier stage of disease may effective against HS (Shivachandra et al., 2011; De Alwis, 1992). In case of outbreaks immediate intravenous administration of these antimicrobials in animals with fever is sometimes effective and rapid systemic bactericidal concentrations can be obtained (De Alwis, 1992). Tang et al. (2009) reported that various antimicrobial drugs such as penicillin, kanamycin, gentamicin, tetracycline, sulphonamides, chloramphenicol and enrofloxacin ceftiofur are effectively used for the treatment of HS disease. Some strains of *P. multocida*, particularly serotype B2, unfortunately exhibit multidrug resistance, particularly against tetracycline and penicillin (Kehrenberg et al., 2001). Due to such high resistance, 100% mortality has been reported in animals suffering in acute clinical symptoms. Thus Shivachandra et al. (2011) suggested that the observation of antibacterial resistance against clinical isolates of *P. multocida* signifies the best way to select the proper drug to control and prevent the severe clinical cases of this disease. In different countries extracts of medicinal plants are considered as potential source of antibacterial agents (Vaou et al., 2021). These plants are main source of various phytochemicals like terpenoids, flavonoids, alkaloids and tannins which have been reported with antimicrobial properties (Lewis et al., 2006; Vaou et al., 2021). Among these medicinal plants *Moringa oleifera* is one known to have vast medicinal values along with immense level of vitamins, minerals and β-carotene, and is originated in Asia, Africa and America (Farooq et al., 2011; Adeyemi et al., 2021). This plant contains phytochemicals having antimicrobial, antipyretic and antioxidant effects (Ajayi and Fadeyi, 2015; Adeyemi et al., 2021). The stem of this plant also has significant curative potentials (thus commonly named as miracle tree) for various types of illnesses and infirmities like influenza, asthma enlarged spleen and liver and inflammation (Zaku et

al., 2015). While the leaves and essential oils of Eucalyptus plants have traditionally been used as herbal remedies due to their antiseptic, antipyretic, and anti-inflammatory properties, there are various applications in daily life (Sabo and Knezevic et al., 2019). There is little or no information on the antibacterial properties of the leaf extracts of *Moringa oleifera* and *Eucalyptus camaldulensis* against *P. multocida*. Hence, current study was hypothesized that extracts of these plants may have inhibitory activities against *P. multocida* isolates. In Pakistan outbreaks of HS disease still found yearly even although many vaccine formulations are commercially available (Moustafa et al., 2013). However, at the onset of outbreak vaccination is not effective to halt the huge mortalities, therefore recognizing HS endemic areas to arrange obligatory vaccination in buffaloes is necessary to control the outbreaks. The present study was also subjected to determine the prevalence of HS in buffaloes of district Shaheed Benazirabad in addition to provide a suitable anti-*Pasteurella multocida* plant extract.

MATERIAL AND METHODS

STUDY AREA AND SAMPLES COLLECTION

A cross sectional study was conducted during the year 2023 in district Shaheed Benazirabad (SBA) formerly known as Nawabshah that is one of the 29 districts of Sindh province. The district is divided into four talukas viz: Nawabshah, Sakrand, Qazi Ahmed and Daur. Samples of blood (n=100) were collected aseptically by jugular vein puncture using sterile syringe from buffaloes suspected of being infected with HS (randomly selected) raised in the above-mentioned areas of the district. However, these suspected animals were identified by clinical findings like swelling in pharyngeal region spreads to ventral neck and brisket or sometimes forelimbs, consequently respiratory distress, terminal recumbence, and high body temperature. Blood samples were immediately transferred into vacutainer tubes containing EDTA, shifted to Department of Veterinary Microbiology, Faculty of Veterinary Sciences, Shaheed Benazir Bhutto University of Veterinary and Animal Sciences Sakrand and refrigerated at 4°C for further microbiological analysis. All study procedures were carried out in line with international standard and were approved by the institute.

ISOLATION AND IDENTIFICATION OF *P. MULTOCIDA*

Blood agar (5percent sheep blood), Nutrient agar and MacConkey's agar were prepared and sterilized according to the procedure described by Karimkhani et al. (2011). Specific pathogens from representative colonies were identified following laboratory methods of plating and isolation. The suspected colonies were further characterized microscopically by using Gram's staining method. Moreover, catalase test, citrate test, glucose, indole test, nitrate test, oxidase test, sugar test and triple sugar iron (TSI) test were

conducted according to procedure described by Jabeen et al. (2013) for the biochemical characterization of *P. multocida*.

PREPARATION OF PLANT EXTRACTS

Leaves of *Moringa oleifera* and *Eucalyptus camaldulensis* plants available at areas of District SBA were firstly washed by distilled water and partially dried on paper towel. After proper drying in oven (60°C), these subsequently were crushed by grinder to make the fine powder. Further the extract was prepared according to the method mentioned by Sasidharan et al. (2011) where, 30ml of double distilled water, 180ml of methanol, and 90ml of ethanol were used to dissolve the powder, the suspension was mixed using shaker (110rpm for 3hrs) and then organic phase was separated from the aqueous phase. The extracts were used against *P. multocida* to analyze the antibacterial activity.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The broth dilution method was applied for the analysis of MIC of *Moringa oleifera* and *Eucalyptus camaldulensis* extract. The plant extract was poured in serial dilution to several tubes containing Mueller Hinton broth in order that the concentrations organized from 2.5 to 0.001mg/ml. The bacterial concentration in these tubes was 5×10^5 CFU/ml. In addition, control tubes were prepared in similar fashion. The test and control tubes were incubated at 37°C for 24 hours. Finally lowest concentration of extract that inhibited the bacterial growth was recorded as MIC.

DISC DIFFUSION METHOD

Sterilized discs of Whatman filter paper No.1 with 6mm diameter were soaked with plant extract, while control discs were prepared with methanol, ethyl acetate and water. After culturing bacteria on Mueller Hinton agar, the discs were placed on the same plates and incubated at 37°C for 24 hours. The diameters of the inhibition zones were measured and compared with those of the controls.

STATISTICAL ANALYSIS

Data was entered and coded in a Microsoft Office Excel 2016 spread sheet. Analyses were performed using statistical software i.e., Statistix 8.1 version, USA through factorial design analysis of variance (ANOVA). Further for critical comparison among the mean values the Least Significant Difference (LSD) test was applied.

RESULTS AND DISCUSSION

PREVALENCE OF HS IN BUFFALO OF DISTRICT SHAHEED BENAZIRABAD:

The current study was carried out to detect the prevalence

of HS in buffaloes and buffalo calves based on clinically suspected animals. Based on clinical signs including elevation of body temperature, edema of throat region, grunting and severe nasal discharge etc., 2244 buffaloes and 576 buffalo calves at district SBA were observed to detect the HS suspected animals. Among the suspected animals the blood samples of randomly selected 100 animals were further cultured, isolated and biochemically characterized. As shown in Table 1, over total number of observed animals the overall prevalence rate was found as 11.10% (249) and 9.20% (53) in buffaloes and buffalo calves, respectively at the district. The highest prevalence was found in buffaloes (19.59%) and buffalo calves (15.87%) of Qazi Ahmed followed by buffaloes (15.14%) of Nawabshah and buffalo calves (11.48%) of Daur talukas. Such area to area variation for the HS prevalence rate had similarly reported by Farooq et al. (2007) who stated that highest prevalence rate of HS was in Gadap (31.10%), Larkana (29.97%), Tharparkar (5.74%), Mirpur (26.64%), and Bhimber (12.86%) and the lowest in Saddar area of Karachi (0.78%) was found. The higher rate of prevalence was also reported in other studies for instance Moustafa et al. (2015) observed 39.8 percent of HS prevalence in Karachi and Kawasaki et al. (2015) found 47.4 percent prevalence in buffaloes of Cambodia. However, Moustafa et al. (2015) stated that the arrival of newly purchased animal with unknown disease statuses, animal overcrowding stress on the farms and animals coming from various areas cause spread of disease and delaying disease control. In case buffalo calves noted considerably susceptible to HS under current study, the reason might be the failure of maternal immunity against the disease at the age of 60 days and vaccination delay (Mahmood et al., 2007), it has also been reported by Kawasaki et al. (2015) that animals if are not vaccinated noted 2.9 times more susceptible to HS disease in contrast to that of vaccinated animals.

In current study typical cultural characteristics of *P. multocida* isolated from buffalo blood samples at district SBA were shown (Table-2) as colonies of whitish in color opaque, circular and translucent in appearance on Nutrient agar, on blood agar whitish in color, opaque, circular and translucent in appearance with no hemolysis were found, whereas on MacConkey's agar the growth was not seen. Further Grams staining of fresh culture showed the organism was noted as Gram negative, short rod or coccid shaped and generally arranged single or in pairs with bipolar appearance. In another study (Nawara et al., 2019) in which the blood samples of rabbits were used to study the *P. multocida* for cultural characteristics of colonies on Nutrient agar, Blood agar and MacConkey agar they found similar observations. While Jabeen et al. (2013) described the cultural characteristics of *P. multocida* from blood samples of cattle as mucoid colonies on Blood agar and gray

Table 1: Prevalence of HS in buffaloes and buffalo calves at district SBA.

Observations	*Talukas of District				Overall
	Nawabshah	Qazi Ahmed	Daur	Sakrand	
No. of buffaloes observed	753	194	524	773	2244
No. of buffaloes suspected **	114	38	49	48	249
Prevalnce rate (%)	15.14	19.59	9.35	6.21	11.10
No. of buffalo calves observed	204	63	122	187	576
No. of buffalo calves suspected **	13	10	14	16	53
Prevelance rate (%)	6.37	15.87	11.48	8.56	9.20

*In each Taluka a total of Ten buffalo farms were examined, **Based on clinical signs HS suspected animals were selected

Table 2: Cultural characteristics of *P. multocida* isolated from buffaloes at district SBA.

Media	Colony characteristics	Microscopic characteristics
Nutrient agar	Whitish Opaque Circular Translucent appearance	Gram negative short rod or coccid structure bipolar
Blood agar	Whitish Opaque Circular Translucent appearance and no hemolysis	
MacConkey's	No colony appearance	

Table 3: Biochemical properties of *P. multocida* isolated from buffalo.

Biochemical tests	Reaction	Inference
Catalase test	Positive	Red or red-violet color in the surface
Citrate test	Negative	Green colony observed
Glucose test	Positive	Fermentation carried out
Indole test	Positive	Tubes contained bubbles
Nitrate test	Positive	Red color appeared on nitrate broth
Oxidase test	Positive	Active budding observed
Sugar test	Positive	For maltose; fawn in color
Triple sugar iron (TSI) test	Positive	Tubes contained bubbles

Table 4: Results of plant extract sensitivity test of *P. multocida*.

Type of extract	Concentration of extract (mg/ml)	Mean inhibition zone (mm)*	No. of samples (over 15)	Sensitive/ resistant
<i>Moringa oleifera</i>	2.50	18.33	13	Sensitive
	2.00	15.33	13	Sensitive
	1.50	14.00	10	Sensitive
	1.00	13.00	11	Sensitive
	0.50	10.67	7	Sensitive
	0.10	9.33	8	Sensitive
	0.05	6.33	4	Resistant
	0.01	Nil	0	Resistant
	0.005	6.33	3	Resistant
	0.001	Nil	0	Resistant

<i>Eucalyptus camaldulensis</i>	2.50	13.00	9	Sensitive
	2.00	9.67	9	Sensitive
	1.50	9.33	6	Sensitive
	1.00	8.33	10	Sensitive
	0.50	8.33	7	Sensitive
	0.10	7.00	9	Resistant
	0.05	6.67	5	Resistant
	0.01	6.33	3	Resistant
	0.005	Nil	0	Resistant
	0.001	6.33	4	Resistant

*The zone size >6.00mm was considered for the growth inhibition

Table 5: Average zone size (mm) inhibited through extracts against *P. multocida*.

Plant extract	Various concentrations (mg/ml)							Overall
	2.50	2.00	1.50	1.00	0.50	0.10	0.05	
<i>Moringa oleifera</i>	18.66 ^a	15.33 ^b	14.00 ^{bc}	13.00 ^c	10.66 ^d	9.33 ^{de}	6.33 ^e	13.55 ^a
<i>Eucalyptus camaldulensis</i>	13.00 ^c	9.66 ^{de}	9.33 ^{de}	8.33 ^{ef}	8.33 ^{ef}	7.00 ^{fg}	6.67 ^{fg}	8.75 ^b
LSD (0.05)	0.7884							0.8533
±SE	1.5961							1.7275

Note: Total 15 samples randomly selected from 100 infected buffaloes were used.

ish on Nutrient agar but no growth on MacConkey agar. In case of Grams staining the results are in line with the observations of Nawara et al. (2019) who stated to organism as Gram negative, cocco-bacillary or short rod shaped and arranged generally in single or pairs with bipolar appearance after staining of fresh culture of the organisms. The findings of Jabeen et al. (2013) are also in accordance with the findings of current study where they found that *P. multocida* was Gram negative and cocco bacillus in shape. In the present study *P. multocida* isolated from buffaloes of district SBA was subjected to further studies for the biochemical properties (Table-3) and results illustrate that catalase test was positive by appearance of active bubbles, glucose test by fermentation, indole test by appearance of red or red-violet color of oily ring on the surface, nitrate test by appearance of red color on nitrate broth, oxidase test observed by active bubbling, sugar (maltose) by appearance of fawn color and triple sugar iron tests by appearance of bubbles in tubes while citrate test noted to be negative by appearance of green colonies. The findings of Nawara et al. (2019) agree with these results reporting that catalase test found positive as bubbles appeared due to production of gas H₂S, Indole test also positive as oily ring appeared on the surface of media when they added Kovacs reagent, they further reported that *P. multocida* did not require citrate as its sole source of carbon and found citrate negative. The results of present study are also matching with the findings of Rashida et al. (2006), and Yami et al. (2017) also found similar biochemical characteristics of *P. multocida*.

EFFICACY OF EXTRACTS (*MORINGA OLEIFERA* AND *EUCALYPTUS CAMALDULENSIS*) AGAINST *P. MULTOCIDA*

In present study the extract of *Moringa oleifera* and *Eucalyptus camaldulensis* each at the concentrations of 2.5, 2.00, 1.50, 1.00, 0.50, 0.10, 0.05, 0.01, 0.005 and 0.001mg/ml were used to compare their efficacy against *P. multocida* (Table-4). The MIC concentration of *Moringa oleifera* was found 0.10mg/ml while it was noted high for *Eucalyptus camaldulensis* (i.e., 0.50mg/ml). However, *P. multocida* was found to be resistant to extract of *Eucalyptus camaldulensis* at concentrations of 0.10, 0.05, 0.01, 0.005 and 0.001mg/ml. Further the extract of medicinal plant *Moringa oleifera* at concentration from 2.50 to 0.10mg/ml inhibited the organism growth by forming zone of a size from 18.33 to 9.33mm in diameter and the organism noted to be sensitive to *Moringa oleifera* while at concentration 0.05, 0.01, 0.005 and 0.001mg/ml it was resistant, and the inhibition zone was formed with a diameter 6.33mm. In case of using *Eucalyptus camaldulensis* extract the organism was found to be sensitive from the concentration 2.50 to 0.5mg/ml by inhibiting the growth with zone size from 13.00 to 8.33mm in diameter while at concentrations 0.10, 0.05, 0.01, 0.005 and 0.001mg/ml it was observed to be resistant to *Eucalyptus camaldulensis* plant extract even forming zone size from 7.00 to 6.33mm. A study conducted by Sabo and Knezevic, (2019) showed 0.07 to 0.5% MIC of *Eucalyptus camaldulensis* against the Gram-positive bacteria but against Gram negative bacteria it was found higher as 0.15-3.2% because they had observed minimum inhibitory concentration for a wide range of bacteria. Further, Pankey and Sabath (2004) stated that the in comparison to Gram

negative, the Gram-positive bacteria are considered more sensitive to *Eucalyptus camaldulensis*, the minimal bactericidal concentrations for most bacteria are equal to or four times higher than the minimum inhibitory concentrations. Moreover, authors indicated that the low range of MIC denotes that *Eucalyptus camaldulensis* act as bactericidal agents. Noteworthy, the inhibitory concentrations varied depend on plant properties, extraction method and organism, being in wide range of 0.08µg/ml to 200mg/ml (Sabo and Knezevic, 2019), for instance crude aqueous leaf extract present lower activity against various bacteria (Abubakar, 2010), whereas bark aqueous of extracts was found highly effective, with minimum inhibitory concentration from 0.1mg/ml to 4.0mg/ml (Mabona et al., 2013). Further, Table-5 shows the comparison between the *Moringa oleifera* and *Eucalyptus camaldulensis* for the growth inhibition of *P. multocida*. Significant ($P < 0.05$) growth inhibition (zone size; 18.66mm) was noted by the extract of medicinal plant *Moringa oleifera* at concentration 2.50mg/ml followed by level 2.00mg/ml (zone size; 15.33mm), 1.50mg/ml (14.00mm) and 1.00mg/ml (13.00mm) in contrast to that of *Eucalyptus camaldulensis* at the level of 2.50, 2.00, 1.50 and 1.00mg/ml where the zone size was measured as 13.00, 9.66, 9.33 and 8.33mm, respectively. However, the plant extract *Moringa oleifera* and *Eucalyptus camaldulensis* at concentrations 0.50mg/ml (zone; 10.66 and 8.33mm, respectively), 0.10 (zone 9.33 and 7.00mm, respectively) and 0.05mg/ml (zone; 6.33 and 6.67mm, respectively) formed markedly lowest inhibition zones against the growth organism. Overall zone size of extract *Moringa oleifera* (13.55mm) was found significantly higher than that of plant extract *Eucalyptus camaldulensis* (8.75mm). *Moringa oleifera* was used for the inhibition of growth of *Enterococcus faecalis* by Arevalo-Hijar et al. (2018) they found 35.5 ± 1.05 mm zone size at the level of 75µg/ml. Such considerable antibacterial activity of *Moringa oleifera* has been supported by Olson and Fahey (2011) who stated that this is due to presence of chemical compound 4-(4-O-acetyl- α -L-rhamnopyranosyloxy) – bezylisothiocyanate in the plant, its mechanism of action involves inhibition of essential cellular membrane enzymes. It is important to note that the current study indicates that with the increase of concentration of both *Moringa oleifera* and *Eucalyptus camaldulensis* the zone size was noted significantly larger. This variation might be due to the high concentration of medicinal plant possess greater quantity of chemical compound to inhibit the growth of organisms (Muhammad et al., 2016). Further present study observed that comparatively the *Moringa oleifera* plant extract inhibited the growth of *P. multocida* significantly higher where the zone size was 13.55 mm than that of *Eucalyptus camaldulensis* extract (8.75mm). The findings of another study support the variation in inhibitory action against *P. multocida* by different plants. They reported that

the plant extract of *Azadirachta indica* (Neem) has a higher capacity to induce cellular lysis of bacteria compared to that of *Moringa oleifera* (Arevalo-Hijar et al., 2018).

CONCLUSIONS

Based on results of present study it could be concluded that among different talukas of Shaheed Benazirabad, the prevalence rate of HS was found higher in Qazi Ahmed followed by Daur, and Nawabshah. The prevalence of HS disease was noted more in young buffalo calves compared to that of buffaloes. The minimum inhibitory concentration of extracts of *Moringa oleifera* and *Eucalyptus camaldulensis* was observed as 0.10 and 0.50mg/ml, respectively. In comparison the efficacy of *Moringa oleifera* was markedly higher than the *Eucalyptus camaldulensis* against *P. multocida*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Barkat Ali Jatoti and Amjad Hussain Mirani conceived and designed the study. Abdul Latif Bhutto, Ambreen Laghari, Abdul Samad Magsi, Ahmed Sultan Jatoti contributed to the analysis of data, laboratory practices and paper writing. Muhammad Mohsen Rahimoon, Aarab Khan Lund participated in overall analyses of the study. Aneela Sultan Jatoti, Om Parkash and Atif Ali Malak collaborated in the sample collection. All authors approved the final manuscript.

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