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Antibacterial activity of successive extracts of some medicinal plants against field isolates of *Pasteurella multocida*

N P USHA[1], K J BIBU[2], SUBIN JOSE[3], A M C NAIR[4], G KRISHNAN NAIR[5] and N D NAIR[6]

Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala 673 576 India

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ABSTRACT

The outbreak of duck pasteurellosis caused by avian strains of *Pasteurella multocida*, which occur during monsoon period, is a serious problem with high mortality and morbidity affecting younger age groups. Medicinal plants reported to have antibacterial action were tested for their efficacy against *Pasteurella multocida* by *in vitro* methods. Successive extraction of whole plant materials of *Leucas aspera*, *Vernonia cineria* and *Ocimum sanctum*, leaves of *Syzigium cumini*, and bark of *Polyalthia longifolia* were performed with petroleum benzine, choloroform, acetone, methanol and water, and were tested for their antibacterial effect using microtitre plate technique (to estimate the minimum inhibitory concentration, MIC) and disc diffusion method (to estimate the zone of inhibition). The results from the above *in vitro* assay revealed the efficacy of different plant successive extracts against duck pasteurellosis.

Key words: Disc diffusion, Microtitre plate dilution, *Pasteurella multocida*, Phytochemical analysis

Duck pasteurellosis, an acute or chronic disease, caused by *Pasteurella multocida (P. multocida)*, with its alarming mortality and morbidity have the potential to become a major bottleneck in the burgeoning of the poultry industry. The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics poses challenges for treating bacterial infections (Walter *et al*. 2011). Even though India is a treasure-trove of medicinal plants, many medicinal plants still remain to be explored to a variety of disease conditions (Bibu *et al*. 2010). The antibacterial activity of many medicinal plants was reported. But use of medicinal plants with antibacterial activity specific to *P. multocida* has not been tested. The present study was aimed at the *in vitro* antibacterial evaluation of the successive extracts of the plants against the field isolates of *P. multocida* which can lead to the idea of evolving cost effective, safe and eco-friendly herbal medicines against duck pasteurellosis.

Present address: [1]Associate Professor and Principal Investigator (ushanpillai@yahoo.com), [2]Ph.D. Scholar, [3]Research Associate, Outreach Programme on Ethnoveterinary Medicine, Department of Clinical Veterinary Medicine, (subinjp@gmail.com), [4]Formerly Professor, Department of Veterinary Pharmacology and Toxicology, (bibujohn@gmail.com), [5]Retired Professor, Department of Veterinary Microbiology, krishnannairg@gmail.com, [6]Professor, Department of Veterinary Pathology, (drdivakarannair @yahoo.com), College of Veterinary and Animal Sciences, Mannuthy.

MATERIALS AND METHODS

Plant materials and successive extraction: Fresh parts of the plant materials were collected from Thrissur District, Kerala, India and were authenticated by Dr Thomas Abraham, Professor, National bureau of plant genetic resources (NPBGR), Regional centre, Thrissur. The plant materials were shade dried and coarsely powdered using an electrical pulveriser. Successive extraction of whole plant materials of *Leucas aspera (L. aspera)*, *Vernonia cineria (V.cineria)* and *Ocimum sanctum (O. sanctum),* leaves of *Syzigium cumini* (*S. cumini*) and bark of *Polyalthia longifolia (P. longifolia)* were performed with petroleum benzine, choloroform, acetone, methanol and water based on their polarity and the yield of the extracts are presented in Table 1.

Bacterial strains used for the study: Pasteurella multocida A: 1 strain (DP1) isolated from a duck farm, serotyped at Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India and maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India was used for the entire study. Purity of the isolate was checked based on morphology, cultural and biochemical characteristics as described by Barrow and Feltham (1993) and the bacterial culture was confirmed as *P. multocida*. Chemicals were procured commercially.

Table 1. Yield (percentage) of successive extracts of plant materials used in the *in vitro* antibacterial analysis against *P. multocida*

Scientific name	Parts used	Yield of successive extracts				
		Petroleum benzine	chloroform	acetone	methanol	aqueous
Leucas aspera	Whole plant	2.205	2.979	0.931	4.156	5.709
Syzigium cumini	Leaves	1.032	2.959	2.21	5.018	8.537
Vernonia cineria	Whole plant	3.051	2.770	1.15	6.023	8.011
Ocimum sanctum	Whole plant	0.895	1.625	0.820	3.669	8.326
Polyalthia longifolia	Bark	5.543	1.887	0.580	9.127	11.695

Antimicrobial assay

Selection of vehicle: Selection of vehicle for the microtitre plate dilution and disc diffusion was done using disc diffusion test (Bauer *et al*. 1966). The following vehicles were used: petroleum benzene, chloroform, acetone, methanol, sterile distilled water, sterile dimethyl sulphoxide (DMSO), Tween 80 and gum acacia. Five millilitre of overnight incubated *P. multocida* culture in tryptone soya broth adjusted with half the concentration of No.1 Mcfarland Nephelometer standard was used for the test. Spreaded *P. multocida* culture in tryptone soya agar plate and kept sterile plain antibiotic disc on the surface of agar. Added 20 µl of each vehicle into the disc and kept for incubation at 37° C for 24 h. Negligible zone of inhibition was observed for Tween 80 and smaller zone of inhibition observed for chloroform. Other solvents did not show any zone of inhibition. DMSO was selected because of its more diffusion power through the agar. The concentration of DMSO to be used as vehicle was determined with tube dilution method. Five millilitre of tryptone soya broth was taken in 5 test-tubes and added various concentrations of DMSO (50 µl,100 µl, 400 µl, 800 µl and 1 ml) and 200 µl each of overnight *P. multocida* culture was inoculated into all the test tubes. All the test tubes were incubated at 37° C for 24 h. The turbidity was observed in all tubes except DMSO with 800 µl and 1 ml. The result obtained revealed that DMSO showed no inhibitory action up to 400 µl.

Microtitre plate dilution technique for the estimation of minimum inhibitory concentration (MIC): The microtitre plate dilution technique was performed using the method of Sheena *et al*. (2003). The plant extracts were dissolved in sterile DMSO to make a total volume of 20 µl and filtered through sterile Whatman syringe filter (0.45 µm). Aqueous extracts were dissolved in sterile distilled water and prepared as above. Various concentrations (200 µg, 500 µg and 1 mg/well) of successive extracts of *L. aspera*, *S. cumini*, *V. cineria*, *O. sanctum* and *P. longifolia* were mixed with 0.05 ml each of bacterial culture (inoculum of 50% transmission at 530 nm in normal saline) and tryptone soya broth to make a volume of 0.18 ml in a 96-well microtitre plate. The plate was incubated at 37° C for 24 h. After the incubation, 0.02 ml of MTT (3–(4,5-dimethylthiazol-2-yl)2,5-diphenyl-2H-tetrazoliumbromide) (5 mg/ml) was added into each well and incubated for 30 min at 37° C. After the incubation period, the inhibition of the growth was detected as colourless wells. The lowest concentration of the extracts that completely inhibited the bacterial growth was assumed as minimum inhibitory concentration (MIC).

Disc diffusion method to determine the zone of inhibition: Disc diffusion method was performed with successive extracts of *L. aspera, S. cumini, V. cineria, O. sanctum* and *P. longifolia* as per Bauer *et al*. (1966). Five millilitre of overnight incubated *P. multocida* culture in tryptone soya broth adjusted with half the concentration of No.1 Mcfarland Nephelometer standard was used for the test. Spreaded *P. multocida* culture in tryptone soya agar plate and kept sterile plain antibiotic disc on the surface of agar.10µl of the extracts were pipetted out into the discs. Standard antibiotic discs of co-trimoxazole and tetracycline were also inserted in a similar fashion. The plates were inverted and incubated for 24 h at 37°C. After 24 h, the zone of inhibition was measured. The experiment was repeated 3 times and the average of the zone of inhibition was taken.

Preliminary phytochemical analysis of the plant extracts: The effective plant extracts obtained with petroleum benzine, choloroform, acetone, methanol and water were tested for the presence of various active chemical constituents namely steroids, alkaloids, tannins, phenolic compounds, flavonoids, glycosides, diterpenes, triterpenes and saponins as per Harborne (1991).

RESULTS AND DISCUSSION

The percentage yield of different successive extracts of plant materials used in the *in vitro* analysis is presented in Table 1. The yield of aqueous extracts of the different plant materials was more than the methanolic, acetone, choloroform and petroleum benzine extracts. The differences in the extract yields might be ascribed to the different availability of extractable components, resulting from the varied chemical composition of plants (Sultana *et al*. 2009).

The results obtained from the microtitre plate technique (Table 2) revealed that the aqueous, chloroform, direct chloroform and petroleum benzine extracts of *S. cumini* and aqueous, chloroform, direct chloroform extracts of *L. aspera* showed MIC at 200 µg. But the methanolic, chloroform, direct chloroform and petroleum benzine extracts of *V. cineria*

Table 2. Minimum inhibitory concentration of successive extracts of plant materials against *P. multocida*

Plants	Extracts	MIC
Leucas aspera	Aqueous	200 µg
	Chloroform	200 µg
	Direct chloroform	200 µg
	Petroleum benzine	500 µg
All the other plant extracts showed no inhibition		
Syzigium cumini	Aqueous	200 µg
	Chloroform	200 µg
	Direct chloroform	200 µg
	Petroleum benzine	200 µg
All the other plant extracts showed no inhibition		
Vernonia cinneria	Methanolic	500 µg
	Chloroform	500 µg
	Direct chloroform	500 µg
	Petroleum benzine	500 µg
All the other plant extracts showed no inhibition		
Ocimum sanctum	Petroleum benzine	200 µg
	Chloroform	200 µg
	Acetone	500 µg
	Methanol	200 µg
	Aqueous	200 µg
Bark of *Polyalthia longifolia*	Petroleum benzine	200 µg
	Chloroform	200 µg
	Acetone	200 µg
	Methanol	500 µg
	Aqueous	500 µg

and petroleum benzine extract of *L. aspera* showed MIC at 500 µg. The petroleum benzine, chloroform, methanol and aqueous extracts of *O. sanctum* showed MIC at 200 µg while its acetonic extract showed MIC at 500 µg. Similarly, petroleum benzine, chloroform, acetone extracts of *P. longifolia* showed MIC at 200 µg; methanolic and aqueous extracts of *P. longifolia* showed MIC at 500 µg. The results (Table 3) obtained for the disc diffusion method revealed that zone of inhibition was exhibited by aqueous, methanolic and acetonic extract of *S. cumini* at 1 mg, 500 µg and 200 µg. The aqueous extract of *O. sanctum* and the acetonic extract of bark of *P. longifolia* exhibited zone of inhibition at 1 mg and 500 µg. None of the other extracts which were used for the study showed zone of inhibition. The results of the antimicrobial assay when analysed with both the techniques revealed that antipasteurellosis effect could be observed with aqueous extract of *S. cumini*, aqueous extract of *O. sanctum* and acetonic extract of bark of *P. longifolia*. The methanolic and acetonic extracts of *S. cumini* also exhibited antipasteurellosis effect in disc diffusion method. The alcoholic and aqueous extracts of *S. cumini* were reported to have antimicrobial activity against a number of pathogenic organisms (Oliveira *et al*. 2007, Gowri and Vasantha 2010).The essential oils and aqueous extract of *O. sanctum* followed by its successive extraction with choloroform and alcohol showed antibacterial activity against gram positive

Table 3. Zone of inhibition of successive extracts of plant materials against *P. multocida*

Plants	Extracts	Dose	Zone of inhibition (diameter in mm)
S. cumini	Aqueous	1 mg	19.0
		500 µg	16.1
		200 µg	14.5
	Methanol	1 mg	18.5
		500 µg	16.0
		200 µg	12.0
	Acetone	1 mg	18.5
		500 µg	16.0
		200 µg	15.0
O. sanctum	Aqueous	1 mg	10.25
		500 µg	8.00
Bark of *P. longifolia*	Acetone	1 mg	12.0
		500 µg	10.0
All other plant extracts did not show any zone of inhibition			
Sulpha trimethoprim			33.2
Tetracycline			27.0

and gram negative bacteria like *E. coli*, *Salmonella* Typhimurium and *Pseudomonas aeruginosa* (Mishra and Mishra 2011). Our results also revealed the antibacterial activity of successive extract of aqueous extract of *O. sanctum* against *P. multocida*, a gram negative organism. In the present study, bark of *P. longifolia* showed antipasteurellosis effect only in microtitre plate technique. Zone of inhibition was not shown by the bark of *P. multocida*. But the acetonic extract of bark of *P. longifolia* showed antipasteurellosis activity in both the antimicrobial assays. Chanda and Nair (2010) reported the antimicrobial activity of leaves of *P. longifolia* against clinically important pathogenic strains. It was evident from the study that some of the extracts having MIC in the range of 200 or 500 µg were negative in disc diffusion technique, i.e no zone of inhibition. On the other hand, extracts such as acetone and methanol extracts of *S. cumini* which had some of the highest diameter of zone of inhibition (in disc diffusion technique), but did not show MIC. The observed discrepancies might be explained by heterogenecity in the pathogenecity of the inocula used for the study.

Preliminary phytochemical analysis of the effective plant extracts that showed the antipasteurellosis effect in both the microtitre plate technique and disc diffusion method revealed the presence of alkaloids, phenolic compounds and tannins in the aqueous extract of *S. cumini* while the aqueous extract of *O. sanctum* revealed the presence of steroids, phenolic compounds, tannins, flavonoids, diterpenes and saponins. Steroids, flavonoids, glycosides and triterpenes were detected in the acetonic extract of *P. longifolia* leaves. Many plants were used because of their antimicrobial properties, which are due to phytocompounds present in them such as phenols (Okoro *et al.* 2010), essential oils (Daferera *et al.* 2003),

terpenoids (Jasmine *et al.* 2011), alkaloids (Omulokoli *et al*. 1997) and flavonoids (Bylka *et al.* 2004). Preliminary phytochemical analysis during the present study also ascertains the presence of biologically active phytochemical constituents, and the antipasteurellosis effect exhibited by the various plant extracts may be attributed to these biologically active phytochemical constituents.

Antibiotics, in purified or chemically modified form, are currently used in the treatment of duck pasteurellosis. The indiscriminate use of these antibiotics has created resistance and none of the commonly used antibiotics against duck pasteurellosis become effective. The objective of using ethnoveterinary medicine comes into effect in this situation. The present *in vitro* study with the medicinal plants will help in evolving cost effective, safe and eco-friendly ethnoveterinary medicine in the treatment of duck pasteurellosis.

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