MOLECULAR IDENTIFICATION AND COMPARATIVE ANTIMYCOPLASMAL ACTIVITY OF THREE INDIGENOUS MEDICINAL PLANTS AGAINST *MYCOPLASMA PUTREFACIENS* ISOLATED FROM SHEEP

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ABSTRACT

Medicinal plants are used for the treatment of human and animal diseases from the ancient time. Screening of medicinal plants for bioactive compound leads to development of new and cheap antimicrobial agents. The present study was carried out to investigate the molecular identification of *Mycoplasma putrefaciens* and to evaluate the antimycoplasmal activity of methanolic extract of three medicinal plants, i.e., *Artemisia herba-alba*, *Calotropis procera* and *Azadirachta indica* against local isolates of *M. putrefaciens* obtained from sheep of different regions of Khyber Pakhtunkhwa, Pakistan. The result showed that significantly ($P < 0.001$) higher number of isolates were obtained from southern (n=43; 14.33%) followed by central zone (n=26; 8.66%). A total of 60 PCR confirmed *M. putrefaciens* isolates (20 from each zone) were subjected to antimycoplasmal activity of plant extracts. The plant extracts were tested against the isolates using agar well diffusion assay by determining the zone of inhibition and minimum inhibitory concentrations (MICs). The results showed that the methanolic extract of *Artemisia herba-alba* exhibited prominent antimycoplasmal activity at 30 mg by producing maximum zone of inhibition (15.4±0.52 mm) against all the tested isolates followed by *Calotropis procera* and *Azadirachta indica* with inhibition zones of 14±0.58 and 11±0.7 mm, respectively. Whereas, the MICs values were 0.03±0.001, 0.3±0.04 and 3.0±0.2 mg/mL for *A. herba-alba*, *C. procera* and *A. indica*, respectively. The results of this study suggest that methanolic extract of all the three indigenous plants exhibited strong antimycoplasmal activity against *M. putrefaciens*, which might be used successfully for the treatment of mycoplasmosis.

Key words: Mycoplasma, sheep, medicinal plants, methanolic extracts, MICs.

INTRODUCTION

The use of plants in treating various diseases is as old as civilization and traditional medicines still provides a major share in treatments of different maladies (Alviano and Alviano, 2009; Fabricant and Farnsworth, 2001). The development of drug resistance issues of antibiotics to various pathogens further signifies the role of herbal medicines. Nowadays, due to historical and cultural reasons, folk medicine is still important in developing countries due to poverty and scarce health services. Therefore, medicinal plants have been extensively used in Unani, Ayurveda and Homeopathic medicine (Kausik *et al.,* 2002). It is estimated that only 1% out of 0.26 million flowering plants on earth have been studied for their bio-active compounds for their medicinal properties (Cox *et al*., 1994; Verpoorte, 2000). The plant extracts are used for their antibacterial, antifungal and antiparasitic properties. Use of herbal medicines are continuously increasing due to their rich source of bioactive compounds, less side effects and also no known resistance issues (Aburjai *et al*., 2001). Screening of medicinal plants for animal infections, especially for caprine antimycoplasmal activity, is neglected chapter. The phyto-chemical compounds after manipulation provide a new and improved drug for the treatment and management of these infectious diseases. Plants are naturally available on every land of the earth, thus are providing cheaper and easily available source for the discovery and development of new drugs. The Khyber Pakhtunkhwa and northern regions of Pakistan are gifted with large reservoirs of flora having high scope for herbal medicines.

Azadirachta indica commonly known as “neem” in subcontinent, which belongs to the family Meliaceae. It is evergreen tree found in most of the tropical countries of the world. The genus Azadirachta, native to India and Burma, is growing in many tropical and semi-tropical regions of the world. The tree is found in Bannu, Dera Ismail Khan districts of Khyber Pakhtunkhwa, similarly it is abundantly present in most parts of Punjab and Sindh provinces. It is a fast growing tree with average height of

15-30 meters. It is used in folk medicine as a principal therapeutic agent in different formulations. About 135 active phyto-chemical compounds like flavonoids, terpenoids, tannins and steroids has been isolated from different parts of neem (Emran *et al*., 2015). The extract of different parts of neem like leaf, bark, seeds and oil showed wide therapeutic indications like antimalarial, anti-inflammatory, antidiabetic and antibacterial (Talwar *et al*., 1997; Biswas *et al.,* 2002; Hoque *et al*, 2007). The U.S. National Academy of Science in a scientific report of 1992 declared "neem a tree for solving global problem". However, very limited research data is available about antimycoplasmal activity of neem plant against different pathogenic species of mycoplasma in animals.

C*alotropis procera* is commonly known as "milk weed" which belongs to family Asclepiadaceae and is consisted of 280 genera and 2000 species. It is widely distributed throughout the world and abundantly found in the tropic and sub-tropic areas of Pakistan. In different studies the ethanolic, methanolic and chloroform extracts of this plant exhibited good antibacterial properties against different pathogens (Ali *et al*., 2014). Its different active compounds are in the form of triterpinoids, alkaloids, resins, calotropin, anthocyanins and proteolytic enzymes in latex, flavonoids, tannins, saponins, mudarin, sterol and cardiac glycosides. Some compounds like terpenes, multiflorenol and cyclisadol have been isolated from the flowers (Nenaah, 2013).

Artemisia herba-alba belongs to the family Asteraceae and is commonly known as white wormwood which consisted of 500 species, and are mainly found widely in the northern hemisphere (Bremer and Humphries, 1993). The Artemisia has different species throughout the world and about 150 species in China and Asia, 175 in Russia, 51 in Japan and 57 in Europe (Shinskin and Bobrov, 1995; Tutin and Persson, 1976). Only about 30 species of Artemisia are investigated for the phytochemical analysis exploring their medicinal uses. The plant of Artemisia is dwarf shrub, commonly grow in FATA and northern regions of Pakistan and also in the western border of Pakistan including the major areas of Afghanistan. It is traditionally used for the treatment of diabetes mellitus, liver diseases, skin infections, anthelmintic, antispasmodic and cancer (Willcox *et al*., 2009; Mohamed *et al*., 2010). The different parts of plants are used for medicinal purposes like the essential oil has antibacterial, antifungal and antigenotoxic effects (Aburjai *et al*., 2001; Bakkali *et al*., 2008). Some important compounds like terpenin, camphor, davonone, herbalbin, flavonoides, acetate and borneol have been isolated from the leaves, flowers, seed, root and stem (Baser *et al*., 2002; Soković *et al*., 2010).

Mycoplasmosis is an important respiratory disease, which is causing heavy losses in small ruminant population throughout the world. Pakistan has large small ruminant population, which is providing a good source of income to the poor farmers and is also earning large amount of revenue for national GDP in the form of meat and hide export. However, this large population of animals is facing various challenges in the form of harsh climatic conditions, infectious and contagious diseases. Among the infectious bacterial diseases mycoplasmosis causes heavy economic losses in the form of high mortality and morbidity. Several pathogenic mycoplasma species are responsible for the respiratory complication in small ruminants. *Mycoplasma putrefaciens* is one of the pathogenic species commonly occuring along with *Mycoplasma mycoides* cluster associated with multiple complications like urogenital infection, joint inflammation, mastitis and respiratory distress (Madanat *et al.,* 2001; Nicholas *et al*., 2008). This disease is contagious in nature that rapidly spreads by direct contact among the herds. The ocular and nasal discharge, excretion from wound and open joints, urine, faeces and milk disseminate the microorganisms in the environment (Lambert, 1987). The disease is traditionally treated with commercially available antimicrobials agents (Nicholas and Ayling, 2003). These agents are also widely used as growth promoters, treatment of infections and prophylaxis as well. However, due to indiscriminate use of these antimicrobial agents, drug resistance issue has been recently developed leading to therapeutic failure (Mathew *et al.,* 2007). This creates an opportunity to explore new drug molecules having high efficacy, low side effects and least chances of drugs resistance. Therefore, the present study was designed to investigate the therapeutic effects of indigenous herbal plants against the local isolates of *Mycoplasma putrefaciens* from sheep.

MATERIALS AND METHODS

Collection and identification of medicinal plants: Fresh leaves of *Azadirachta indica*, *Calotropis procera* and aerial part of *Artemisia herba-alba* were collected from Peshawar, Dera Ismail Khan and Abbottabad, respectively. All the species of plants were identified by Herbal taxonomist at Pakistan Forest Institute, Peshawar. Leaves of collected plants were thoroughly washed with tap water followed by final dip in the distalled water. The washed clean leaves were dried in shade for 15 days. The clean dried leaves were grinded to fine powder by electric grinder (Moulnix, 600 W LM-240 France). Approximately 100 g grinded powder from each plant was separately placed in 1000 mL of absolute methanol for 4 weeks with regular shaking. The extracts were then filtered through muslin cloth followed by Whatmann filter paper No.1. The methanol was removed under rotary evaporator. The dry extract was kept in air tight container at 4 ^{o}C till further use. The plant extracts were

dissolved in 10% DMSO (Sigma-Aldrich) then kept in water bath for 30 minutes at 40 ºC for proper dissolution of all the bio-active components. The final concentration of each extract were prepared as 05, 10, 20 and 30 mg/mL for *in vitro* study.

Isolation and identification of *Mycoplasma putrefaciens:* For isolation of *M. putrefaciens* the samples were collected from sheep suffering from respiratory syndrome in the three different climatic regions of Khyber Pakhtunkhwa. A total of 900 nasal swabs (300 from each climatic zone) northern, central and southern were collected from animals suspected for respiratory and mixed infections. The samples were taken by sterile cotton swab and then transferred to the transport media the PPLO broth (Difco™, Sparks, MD, USA). Samples were incubated in anaerobic incubator (5% CO_2) at 37 ºC for 3-7 days. Those samples showing turbidity and whirling movement were recultured on agar media for the growth of typical mycoplasma colonies.

DNA extraction and PCR amplification: The positive cultures of Mycoplasma species were subjected for DNA extraction by Trizol reagent (TRIREAGENT®) method according to the manufacturer's directions. The species specific primers SSF1-F (5'-GCG GCA TGC CTA ATA CAT GC-3'), SSR1-R (5'-AGC TGC GGC GCT GAG TTC A-3') were used for the molecular identification of *Mycoplasma putrefaciens* as described previously (Shankster *et al*., 2002).

Preparation of *M. putrefaciens* culture: The inoculum was prepared by suspending the overnight colonies of *M. putrefaciens* taken from PPLO (Difco™, Sparks, MD, USA) agar media and transferred in fresh PPLO broth maintained in sterile glass tube and adjusted the bacterial growth to 10^3-10^4 CFU/mL.

Determination of antibacterial activities: The antibacterial activities of plants extract against the isolates were carried out by the following methods.

Determination of minimum inhibitory concentrations (MICs): The micro broth dilution test was performed as described previously (Hanan, 2000). The minimum inhibitory concentrations (MICs) of methanolic extract were carried out in 96 well micro titration plates. The PPLO media volume, inoculum and plant extracts used in this method were selected according to Neal *et al.* (2012). After making dilution of 30 µL plant extract, 10 µL broth cultures containing 10^3-10^4 CFU/mL organisms of *M. putrefaciens* was added to all wells expect the sterility control. From the freshly prepared PPLO broth 200 µL was added to each well of properly labelled and sterilized micro-titration plate. Plant extracts were added to the micro-titration plate in triplicate to make a final concentration of 30 mg/mL in the first wells and then serially diluted 10 folds to make further concentrations, i.e., 3.0, 0.3, 0.03 and 0.003 mg/mL of each extract. Tylosin tartrate (1.0 mg/mL) was used as standard drug and added to three designated wells of the micro-titration plates. Ten µL of the washed bacterial culture (OD_{600}= 0.3) was pipetted to each well of the micro titration plate except the negative control. The Optical density (Growth of the bacteria) was checked at 600 nm through ELISA reader (Humareader Plus, 3700 Human, GmbH, Germany) before incubation (t = 0) and 48 hours (t = 48) after incubation at 37 ˚C with 5% CO_2.

Agar well diffusion assay: The PPLO agar plates were streaked with 10 uL of *M. putrefaciens* culture containing 10^3-10^4 CFU/mL. The inoculated agar was then punched with sterile cork borer to make six different 6 mm open well on agar surface. The open wells were poured with different plant extract having concentration of 30, 20, 10, 05 mg/mL. Dimethyl sulfoxide (DMSO) 10% was used as negative control while tylosin tartarate (Selmore, Pakistan) 1.0 mg/mL as a positive control. All these activities were carried out in Biosafety cabinet to avoid contamination. The agar plates were then incubated in 5% CO_2 incubator at 37 ºC for 24-48 h. The zone of inhibition was recorded in millimeter (mm) around each tested plant concentrations after 48 h. For comparison negative control only DMSO 10% without any antimicrobial agent was used. Plates were made in triplicate.

Statistical analysis: Data was compiled in Microsoft Excel and analyzed through SPSS 19.0 software to check for statistical significance. Z-test was used to check significant proportion (percent) difference between the *M. putrefaciens* isolates and different climatic zones. The student-t test and one way ANOVA was used to check statistical difference between different treatments used for anti-mycoplasmal assay. Least Significant Difference (LSD) test was used to separate the means that were significantly different. $P \leq 0.05$ was considered as significant.

RESULTS

Out of total 900 cultured samples 176 (19.55%) showed mass turbidity and whirling movement in PPLO broth, which indicated positive growth of Mycoplasma. The zone wise isolation was 63 (21%), 92 (30.67%) and 121 (40.34%) from northern, central and southern zones, respectively. The positive growth were sub cultured on PPLO agar media and out of the total samples 118 (13.11%) produced typical Mycoplasma colonies (**Fig. 1**).

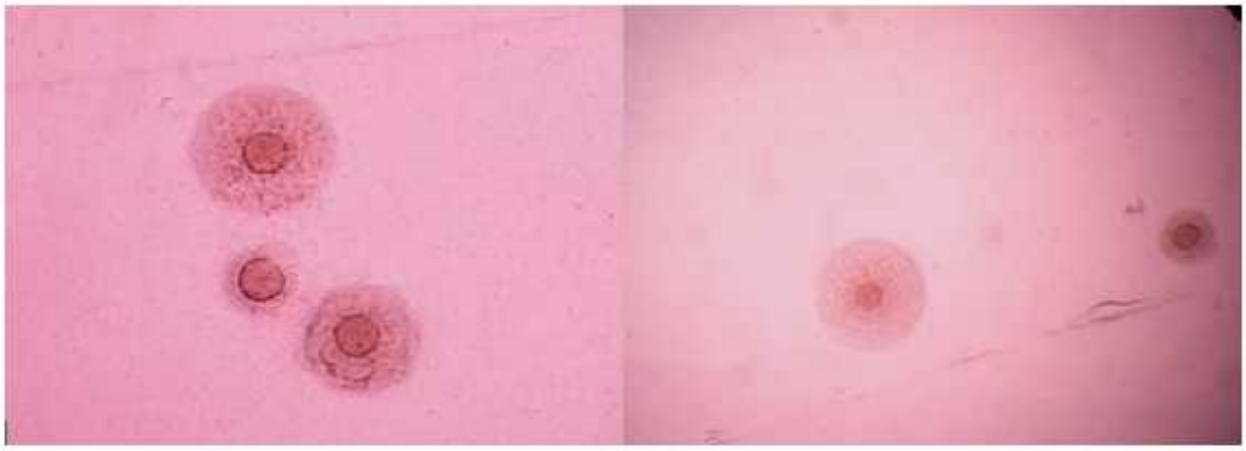

Fig.1 Typical *Mycoplasma putrefaciens* colonies on PPLO agar, isolated from sheep in Kohat Southern zone of KPK, Pakistan, magnification at 4X.

Of total samples 87 (9.66%) were identified as *M. putrefaciens* by using species specific PCR analysis with amplicon size of 540 bp (Fig. 2).

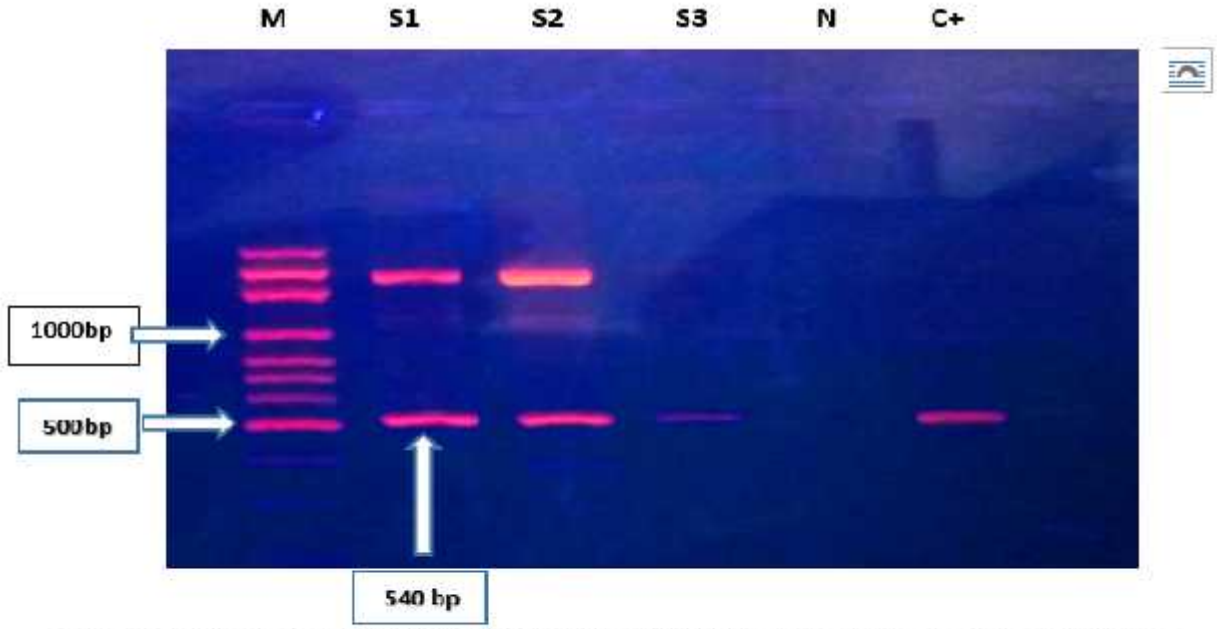

Fig 2. PCR product of *Mycoplasma putrefaciens* isolated from sheep suffering from respiratory syndrome with an amplicon size of 540 bp.
Lane-M =DNA ladder (100 bp), lane S1-3=field isolates, lane-N=negative control and Lane -C^+ – positive control.

The results revealed that 18 (6%), 26 (8.6%) and 43 (14.33%) of suspected samples were confirmed positive for *M. putrefaciens* from northern, central and southern zones, respectively. Data was analyzed by Z-test to check significant proportion (percent) difference between the *M. putrefaciens* and climatic zones. The prevalence of *M. putrefaciens* in different zones and the proportion differences between the zones were tested and the results are displayed in Table 1A and 1B. It is evident that in comparison to all three zones, the maximum prevalence was observed in southern zone followed by central zone, while minimum prevalence was recorded in northern zone. In addition, the *P*-value of the test for proportion difference between the zones for *M.*

putrefaciens prevalence indicated that it was significantly lower ($P < 0.05$) in northern zone as compared to southern and central zones. However, no significant differences in the prevalence of *Mycoplasma putrefaciens* were noted in northern and central zones of Khyber Pakhtunkhwa, Pakistan.

Table-1A. PCR confirmed *Mycoplasma putrefaciens* isolated from sheep in different climatic zones of Khyber Pakhtunkhwa, Pakistan.

Climatic Zones	Status		Total
	Positive (%)	Negative (%)	
Northern	18 (6)	282 (94)	300
Central	26 (8.6)	274 (91.33)	300
Southern	43 (14.33)	257 (85.66)	300
Total	87 (9.66)	813(90.33)	900

Table- 1B. *Mycoplasma putrefaciens* significance of difference between proportions (percent) across different climatic zones of Khyber Pakhtunkhwa, Pakistan.

Pairs	Proportional Difference	Z- value	P- value
Northern vs Central	-0.02	-1.2	0.21^{NS}
Northern vs Southern	-0.083	-3.38	0.001^{**}
Central vs Southern	-0.056	-2.18	0.02^{*}

*Significant= P< 0.05, **= highly significant, NS= non-significant

The antimycoplasmal activity of methanolic extract of the three plants by using agar well diffusion assay is presented in **Table 2**. *Artemisia herba-alba* showed a maximum inhibition zone of 15.4±0.52 mm at 30 mg/mL followed by *Calotropis procera* and *Azadirachta indica* with 14±0.58 mm and 11±0.72 mm zone of inhibition, respectively. It was also evident from the results that methanolic extract of all the three plants produced zone of inhibition at all concentration levels, higher concentrations produced wider zone of inhibition. The maximum antimycoplasmal activity produced by tylosin tartrate was 17±0.65 mm, which was kept as positive control, while no inhibitory zone was recorded around DMSO.

Methanolic extract from leaves of the tested plants were further analyzed by micro broth dilution method to determine the minimum inhibitory concentrations (MICs) as shown in **Fig. 3**. MICs for different extracts were found 0.03 mg/mL, 0.3 mg/mL and 3.0 mg/mL for *Artemisia herba-alba*, *C. procera* and *A. indica*, respectively. The tylosin tartrate was used as a positive control having MIC of 0.002 mg/mL.

Table 2. Antimycoplasmal activity of methanolic extract of *A. indica, C. procera & A. herba-alba* using agar well diffusion assay against *Mycoplasma putrefaciens*.

Plant species	Concentration (mg/mL)	Diameter zone of inhibition (mm)		
		Zone of inhibition (mm)	Tylosin tartrate C^{+}	DMSO C^{-}
Calotropis procera	05	$4±0.32^{f}$	17±0.60	-
	10	$5.8±0.37^{e}$		-
	20	$11.6±0.51^{c}$		-
	30	$14±0.58^{b}$		-
Azadirachta indica	05	$2±0.44^{gh}$		-
	10	$4.4±0.5^{f}$		-
	20	$6.8±0.37^{de}$		-
	30	$11±0.72^{c}$		-
Artemisia herba-alba	05	$3±0.32^{g}$		-
	10	$7.6±0.50^{d}$		-
	20	$12±0.32^{c}$		-
	30	$15.4±0.52^{a}$		-

Means with different superscripts in the column are significantly different at α=0.05; LSD =1.32.

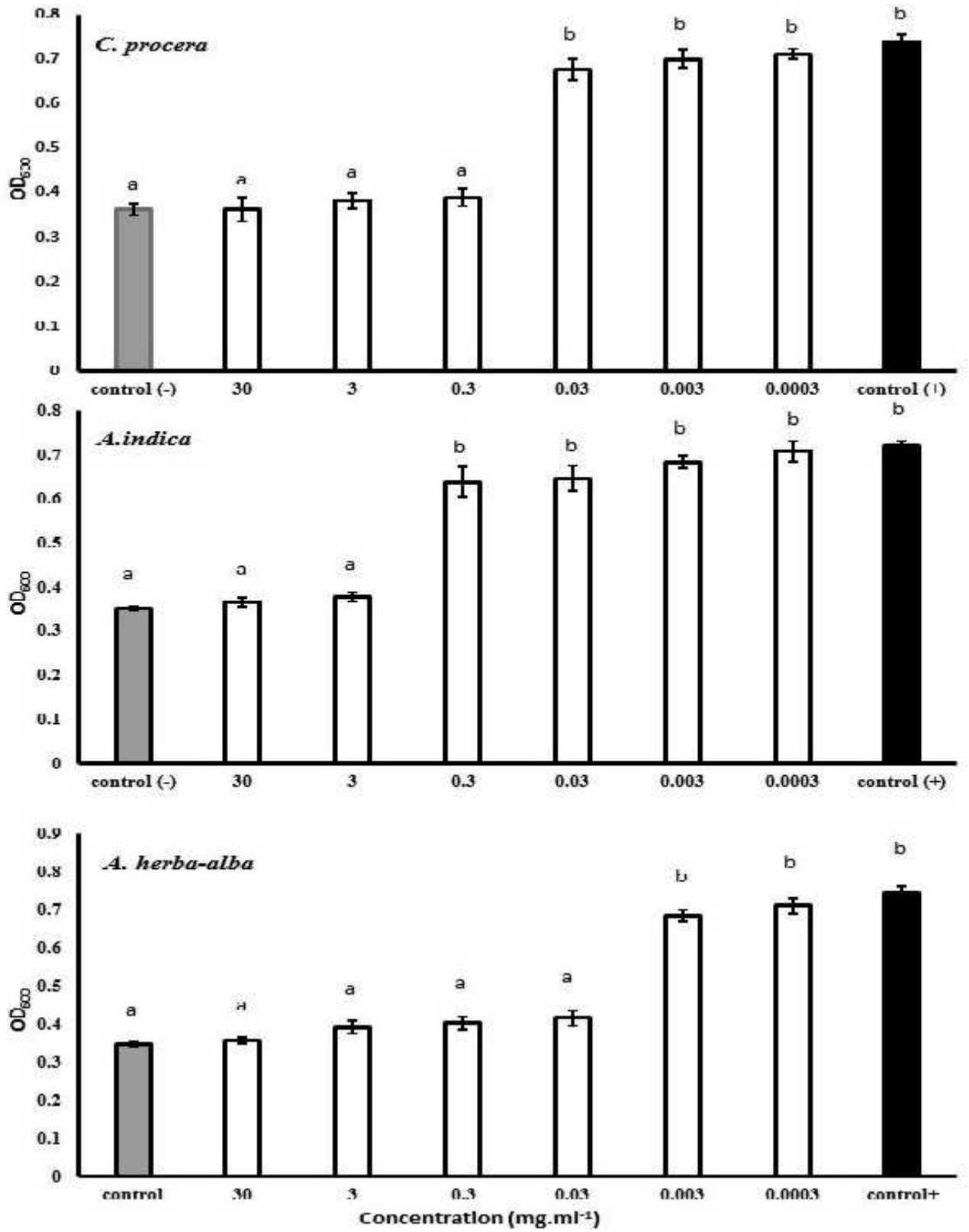

Fig 3. Antimycoplasmal activity of methanolic extract of *Azadirachta indica, Calotropis procera* and *Artemisia herba-alba* against local isolates of *Mycoplasma putrefaciens* using micro broth dilution method.

Different letters indicate statistically significant differences (at α =0.05).

DISCUSSION

Mycoplasma species are normal inhabitant of nasal and respiratory mucosa of animals. Severe stress conditions and immunosuppression make the animals susceptible to various pathogenic microorganisms including the mycoplasma species to produce clinical complications. Several pathogenic species of mycoplasma having respiratory tissue tropism lead to severe respiratory syndrome in small ruminants. The disease is treated by different commercially available antimicrobials with different outcomes. In the underdeveloped countries the indiscriminate use of different antibiotics in veterinary practices are responsible for development of drug resistance that ultimately leads to treatment failure. To avoid and address the drug resistance problems the medicinal plants therapy is getting importance in the recent era. Development of new antimicrobial agents from plants could be useful in combating the emerging resistant species of microbes with improved efficacy, least side effects and high level of safety (Srivastava and Kumar, 2000). The results obtained from the present study confirmed that the local isolates of *M putrefaciens* were sensitive to the tested methanolic plant extracts. The *Calotropis procera* showed moderate antimycoplasmal activities both in well diffusion assay as well as micro broth dilution method. Our results were in agreement with the findings of Mako *et al*. (2012), they used leaf and root extracts of *C. procera* as an antibacterial agent at different concentrations against different bacteria. Similar findings were also reported by (Shittu *et al*., 2004), who confirmed that leaves of *C. procera* have stronger antibacterial activity than roots. The antimicrobial activity of *C. procera* may be due to the presence of bioactive compounds like calotropains, mudarin, glycosides, flavonoides and calactin (Parrotta, 2001; Nenaah, 2013).

The extract of *A. indica* has wide range of therapeutic properties like anti-inflammatory, antimalarial, antifungal and antibacterial (Hoque *et al*., 2007). It proved that the plant of *A. indica* has strong antimicrobial activities; however, less research work has been carried out on sensitivity against pathogenic *Mycoplasma* species. The presence of bioactive compounds like azadirachtins, limonoids, azadirone, nimbidin, nimbidinin, nimbinin, nimbidic acid, quercetin and *β*-sitosterol, flavonoids, terpenoids, carotenoids in *A. Indica* leaves might be responsible for strong antibacterial and antifungal activity as compared with bark and seed (Verkerk and Wright, 1993Subapriya and Nagini, 2005). Similarly, in another study it was revealed that the bark extract showed antibacterial activities at all concentration used against *Pseudomonas aeruginosa*, *Corynebacterium diphteriae* and Bacillus species (Yerima *et al*., 2012).

The findings of present study revealed that among the tested plants *Artemisia herba-alba* was found the most effective antimycoplasmal agent as compared to other tested plants. It inhibited the growth of *Mycoplasma putrefaciens* at all concentrations of 30, 20, 10 and 05 mg/mL. The results are in agreement with the findings of (Al-Momani *et al*., 2007), who demonstrated that methanolic extracts of Jordanian plants of *Artemisia herba-alba* showed strong antibacterial activity against 32 isolates of pathogenic Mycoplasma species. In the present study *Artemisia herba-alba* showed the lowest MIC value of 0.03mg/mL against all the tested isolates. The lowest MIC value of *Artemisia herba-alba* 0.05 and 0.12 mg/mL against different gram positive and negative bacteria was also previously reported (Peda *et al*., 2015). The antibacterial activities might be due to some phyto-active compounds like davonone, herbalbin, flavonoides, acetate and borneol (Baser *et al*., 2002).

Conclusion: The present study has highlighted the antimycoplasmal effects of three indigenous medicinal plants extract the *Azadirachta indica*, *Calotropis procera* and *Artemisia herba-alba* against the local isolates of *M. putrefaciens* responsible for respiratory syndrome of small ruminants. The results of this study suggest that methanolic extract of all the three indigenous plants exhibited strong antimycoplasmal activity against *M. putrefaciens* that might be used for the treatment of ruminant mycoplasmosis. These findings also reflect successful approach in the direction of new antimycoplasmal drug discovery from indigenous herbal plants in veterinary practices to treat and manage multi-drug resistant against fetal infections.

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