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Evaluation of methanolic extract of *Allium sativum* and *Saussurea costus* in yaks with infectious keratoconjunctivitis

SAMIRAN BANDYOPADHYAY[1], T K BISWAS[2], D SASMAL[3], I SAMANTA[4] and M K GHOSH[5]

National Research Centre on Yak, ICAR, Dirang, West Kameng, Arunachal Pradesh 790 101 India

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ABSTRACT

The present study was undertaken to evaluate the efficacy of crude methanolic extract of fresh bulb of *Allium sativum* (G-MeOH), and roots of *Saussurea costus* (SC-MeOH) in yaks (*Poephagus grunniens*) with infectious keratoconjunctivitis. Bactericidal activity of G-MeOH and SC-MeOH was evaluated against *Moraxella bovis* and *Neisseria* spp isolated from eyes of yaks and yak hybrid with infectious keratoconjunctivitis and flies (*Musca domestica*). Following single disc diffusion assay, 5 resistant isolates of both bacteria were included for further study. Single disc diffusion and broth dilution method were used to determine the inhibitory effect of G-MeOH and SC-MeOH. Both the extracts displayed dose dependent antibacterial effect against all the isolates. Thereafter, the diluted plant derived products were subjected to clinical trials. Yaks (18) with infectious keratoconjunctivitis were included for clinical trial. Following application of the extracts, 8 yaks out of 12 (5 cured by G-MeOH, 3 cured by SC-MeOH) were completely cured within 7 days. However, 6 yaks where no extract was applied did not show any recovery. G-MeOH (83.33%: 5/6) exhibited the most prominent clinical efficacy followed by SC-MeOH (50%: 3/6). The results supported the traditional wisdom of herbal remedy use and suggested a potential clinical testing for G-MeOH and SC-MeOH on infectious keratoconjunctivitis.

Key words: Antibacterial activity, Costus, Garlic, Keratoconjunctivitis, *Moraxella bovis*, *Neisseria* spp, Yak.

Yak (*Poephagus grunniens*) is a unique bovine species of high economic importance in high hill and snow bound areas of India, China, Mongolia, Bhutan, Nepal and Russia (Bandyopadhyay and Bhattacharya 2008). Apart from typical inherent problems of yak husbandry several diseases were also found responsible for diminishing yak population of India (Bandyopadhyay *et al.* 2008a, Bandyopadhyay *et al.* 2008b).

Infectious keratoconjunctivitis (pink eye) is the most common ocular problem encountered in Indian yaks (Bandyopadhyay *et al.* 2007a, Bandyopadhyay *et al.* 2007b, Bandyopadhyay *et al.* 2008 c). *Moraxella (Branhamella) bovis* was reported to be the main causative organism in association with *Neisseria, Chlamydia* and *Mycoplasma* (Radostits *et al.* 2000). The conventional antibiotic therapy against these pathogens is very expensive for the poor tribal farmers. Moreover, the pathogens are not always responsive to conventional antibiotics due to emergence of multiple drug resistance (Radostits *et al.* 2000). A safe, cost-effective, alternative therapeutic strategy against these microbial pathogens especially from herbal origin is the need of the hour.

Garlic (*Allium sativum*) has been documented for its antibacterial properties against various microbial pathogens (Ahshan *et al.* 1996, Ankari and Mirelman 1999). *Saussurea costus,* commonly known as 'costus', is usually found in moist shady areas in brich forests of East Asia. The root of the plant is reported to be used as tonic, stimulant and antiseptic in traditional Chinese and Tibetan medicine (Yeung 1985, Tsarong 1994). The present investigation describes about the effect of methanol extract of *A. sativum* (G-MeOH) and *S. costus* (SC-MeOH) on the growth and viability of *Moraxella bovis* and *Neisseria* spp *in vitro*. It also elaborates the clinical efficacy of these extracts in the yaks with infectious keratoconjunctivitis.

MATERIALS AND METHODS

Collection, culture and maintenance of bacterial isolates: Moraxella bovis, Neisseria spp were isolated from the eyes of yaks and yak hybrids with clinical manifestation of infectious keratoconjunctivitis and flies (*Musca domestica*) abundant in the regions. The samples were cultured in nutrient broth and subsequently in trypticase soy agar and blood agar at 37°C overnight. The isolated colonies were identified by

Present address: [1]PhD Scholar, Department of Clinical Veterinary Medicine, Ethics and Jurisprudence e-mail: isamanta76@gmail.com, [2]Scientist, [3]Subject Matter Specialist, [5]Sr Scientist.

[4]Assistant Professor, Department of Veterinary Microbiology, West Bengal University of Animal & Fishery Sciences, 37, K B Sarani, Kolkata – 700 037.

colony structure, haemolysis, bipolar staining and standard biochemical tests (Bandyopadhyay *et al.* 2008c).

In vitro antimicrobial drug sensitivity profile of the isolates: The bacterial isolates recovered from yak were subjected to *in vitro* drug sensitivity assay following the single disc diffusion method (Bauer *et al.* 1966). The antibiotics used in the assay were cifrofloxacin (10 µg), colistin (10 µg), co-trimoxazole (25 µg), gentamicin (10 µg), nitrofurantoin (300 µg), streptomycin (10 µg), tetracycline (30 µg), ampicilin (10 µg), cephotaxime (30 µg), cephalexin (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), furazolidone (50 µg), norfloxacins (10 µg) and oxytetracycline (30 µg). For each bacterium 5 resistant strains were included for further *in vitro* study.

Plant materials and extract preparations: Fresh bulbs of *A. sativum* were collected from the local market and fresh roots of *S. costus* were collected from Tawang Botanical Nursery, Tawang, Arunachal Pradesh, India. The bulbs and roots were shade dried and finely chopped. The chopped materials were suspended in petroleum ether at 4°C overnight to remove the lipid substances. The supernatant was discarded and residue was again dried at room temperature. The dried residue was further suspended in methanol under refrigeration overnight. The supernatant was collected and filtered through Whatmann No. 1 filter paper. The filtrate was dried in rotational vacuum concentrator to evaporate the methanol. The stock solution was prepared by dissolving the extract in dimethyl sulfoxide (DMSO) at 200 mg/mL. Each time a fresh stock solution was prepared for the assay of *A. sativum* (G-MeOH) and *S. costus* (SC-MeOH).

Determination of antibacterial effect of methanolic extract (MeOH): Sterile test tubes containing 10 mL of trypticase soy broth were supplemented with different concentrations of G-MeOH and SC-MeOH (ranging from 0.00–12.0 mg/mL for G-MeOH and 0.00–30 mg/mL for SC-MeOH). The tubes were inoculated separately with 5 multi-drug resistant strains of *M. bovis* and *Neisseria* spp at a concentration of 1.8 $\times 10^6$/mL, 2.5 $\times 10^6$/mL respectively. The initial OD (optical density) was observed at 650 nm. Following incubation of the organism for 24 h again the OD_{650} was observed. The growth and viability of the organisms was calculated in terms of turbidity using UV spectrophotometer. The difference between final OD_{650} and the initial OD_{650} (specific OD_{650}) is interpreted as the growth of the bacteria. The viability of bacteria at any specific concentration was calculated as the percentage of specific OD_{650} at that concentration versus the specific OD_{650} of the tube without *A. sativum* and *S. costus* supplementation (negative control). The calculation can be shown as follows

$$\text{Percent viable} = \frac{\text{Specific } OD_{650} \text{ (final } OD_{650} - \text{initial } OD_{650}) \text{ of any concentration of MeOH} \times 100}{\text{Specific } OD_{650} \text{ (final } OD_{650} - \text{initial } OD_{650}) \text{ of the negative control (MeOH)}}$$

For each concentration of *A. sativum* and *S. costus*, equal amount of DMSO was added to each tube to exclude out the bactericidal effect of the vehicle, if any.

Determination of antibacterial effect of methanolic extract (MeOH) using single disc diffusion method: Further, standard disc diffusion method was employed with little modification. Briefly, sterile discs were impregnated with each of the extract (200 mg/mL) to get the desired concentration. The discs were dried under laminar flow cabinet overnight. Following spreading the bacterial inoculums (Resistant strains of *M. bovis* and *Neisseria* spp as stated above) evenly over the surface of Muller Hinton agar plates, the impregnated discs were positioned and incubated at 37°C for 24 h. Sterile disc was used as negative control. The antibacterial activity was interpreted from the size of the diameter of zone inhibition measured to the nearest millimeter (mm) as observed from clear zones surrounding the discs.

Dilution of the stock solution for in vivo *trial:* The stock solution of 200 mg/ml of *A. sativum* (G-MeOH), and *S. costus* (SC-MeOH) was diluted further in sterile double distilled water at various concentrations (1–50 mg/ml). The diluted solutions were applied on the eyes of the swiss albino mice thrice daily consecutively for 7 days to observe for adverse effect, if any. The aliquots were preserved at –20°C for further use.

Clinical trial in yaks with keratoconjunctivitis: Yaks (18) from the farm of the institute with overt and typical clinical manifestation of infectious keratoconjunctivitis like unilateral or bilateral conjunctivitis, keratitis, profuse ocular discharge, photophobia and corneal opacity were included for the study. Bacterial culture isolated from ocular swabs was maintained on nutrient agar slant/trypticase soy agar slant and were conventionally identified as described earlier. The yaks (18) were divided in 3 groups with 6 animals each. Two groups were tested with G-MeOH and SC-MeOH. In the infected eyes, the extracts were administered @ 0.5 ml/eye 4-times daily (at the concentration of 30 mg/mL), for 7 days. Another group was kept as vehicle control where 0.5 ml of 0.5% DMSO (v/v) in water was applied.

Statistical analysis: The results were expressed as the mean±SE of the results of 5 isolates in duplicate. MIC_{50} and MIC_{80} were determined by fit spline/lowness using Graph-pad prism software version 4.0.

RESULTS AND DISCUSSION

Infectious keratoconjunctivitis associated with *Moraxella bovis*, *Neisseria* spp. was found wide spread among the yaks in India. The problem increased substantially during autumn and spring. This was probably due to abundance of flies (*Musca domestica*) and increased intensity of sunlight during these seasons. Flies and intense UV irradiation were reported as important predisposing factors for keratoconjunctivitis apart from microorganisms (Akerstedt and Hofshagen 2004).

Large hair growth around the eyes is also an important contributing factor due to constant irritation (Bandyopadhyay *et al.* 2007a). The wide spread presence of the disease can be attributed to the way yak husbandry is practised in India. Yaks are generally reared under transhumance system of migration and during lean season they come down to lower altitude in search of feed. During this period, they share common grazing land, falls and streams with other domestic and wild animals like hill cattle and mithun (Bandyopadhyay *et al.* 2007a, Bandyopadhyay *et al.* 2008b, Bandyopadhyay *et al.* 2008e). Moreover, yaks are kept in herds of 50–200 animals. Thus increased contact between animals provides an easy avenue for the transmission of pathogen (Bandyopadhyay *et al.* 2008b, e).

M. bovis and *Neisseria sp* were resistant to colistin, co-trimoxazole, gentamicin, nitrofurantoin, streptomycin, ampicilin, cephalexin, nalidixic acid, furazolidone. Till date, very limited literature is available on the multi-drug resistance profile of *M. bovis* and *Neisseria* sp. However, the increased use of antibiotics both as a growth promoter and in disease management exposes these pathogens to low and sub-lethal concentrations of these agents and indirectly helps them to evolve novel resistance mechanism (Williams *et al.* 1989). Overall antibiogram profile of this study reflects that this resistance feature is remarkably increased when the isolates were taken from the hosts (yaks and hybrids) rather than from carriers (flies). This may be due to extensive and indiscriminate use of antibiotics in these animals. The exploration of muti-drug resistant pathogens in the present study is quite alarming and strongly indicates towards the judicious use of antimicrobials in clinical application.

To study the antibacterial efficacy of *A. sativum* (G-MeOH) and *S. costus* (SC-MeOH), strains of *M. bovis* and *Neisseria* spp were selected on the basis of their antibiogram profile. Isolates resistant to more than 5 antibiotics were included for this study. The effect of G-MeOH and SC-MeOH on the bacterial viability is depicted in Tables 1 and 2 respectively. G-MeOH and SC-MeOH at a concentration of 7.57 mg/mL and 7.516 mg/mL, respectively, caused a marked decline (80%) of the viability of the resistant strains of *M. bovis* (Table 1). Strains of *Neisseria* were also sensitive at 6.14 mg/mL and 28.996 mg/mL concentration of G-MeOH and SC-MeOH respectively. The disc diffusion assay revealed (Table 2) comparable zones of inhibition with G-MeOH (10–15 mg/disc) and SC-MeOH (30 mg/disc) against *Neisseria* spp and *M. bovis* at 24 h of incubation. G-MeOH and SC-MeOH reduced the growth of *M. bovis* and *Neisseria* spp conspicuously. Control discs had no observable effect on the bacterial growth. It is clear that the extracts suppressed the growth of multi-drug resistant isolates of *M. bovis* and *Neisseria* spp, recovered from the eyes of yaks and yak hybrid with infectious keratoconjunctivitis or pink eye, in a concentration-dependent manner using broth dilution method. Similar profile was also observed in disc diffusion assay. This may be due to the fact that G-MeOH and SC-MeOH mediate its antibacterial property in different mechanisms than the antibiotics.

G-MeOH and SC-MeOH at the concentration of 30 mg/mL was subjected to *in vivo* trial. This concentration was more than the MIC_{80} value of both herbal extract tested against all the isolates. Moreover, above this concentration swiss albino mice started showing adverse reaction like restlessness, irritation etc. when applied on their eye. For clinical trial 18 yaks with overt clinical and microbiological evidence of infectious keratoconjunctivitis were included for the study. *M. bovis* and *Neisseria* spp were detected in the conjunctival swab of all the 18 yaks with infectious keratoconjunctivitis. Twelve yaks (6 in each group) were tested with extracts of G-MeOH and SC-MeOH, @ 0.5 ml/eye/4 times daily (at the concentration of 30 mg/mL) for

Table 1. MIC_{50} and MIC_{80} of methanolic extract of *A. sativum* (G-MeOH) and *S. costus* (SC-MeOH) against 5 different multidrug resistant isolates of *Moraxella bovis* and *Neisseria* spp (data shown as mean ± SEM; *n* = 5 different isolates in duplicate)

Organism	G-MeOH			SC-MeOH		
	5 mg/disc mm	10 mg/disc mm	15 mg/disc mm	10 mg/disc mm	20 mg/disc mm	30 mg/disc mm
Neisseria	17.6 ± 0.59	24.2 ± 0.21	27.8 ± 0.79	13.4 ± 0.51	17.8 ± 0.62	21.9 ± 0.17
M. bovis	13.8 ± 0.35	20.9 ± 0.67	28.9 ± 0.36	18.3 ± 0.71	21.8 ± 0.53	29.6 ± 0.42

Table 2. Evaluation of G-MeOH and SC-MeOH against *M. bovis* and *Neisseria* spp by disc diffusion method (data shown as mean ± SEM; *n* = 5 different isolates duplicate)

Organism	G-MeOH		SC-MeOH	
	MIC_{50}(mg/mL)	MIC_{80}(mg/mL)	MIC_{50}(mg/mL)	MIC_{80}(mg/mL)
Neisseria	2.63 ± 0.65	6.14 ± 0.55	12.201 ± 0.92	28.996 ± 0.69
M. bovis	3.96 ± 0.42	7.57 ± 0.38	4.513 ± 0.71	7.516 ± 0.57

seven days. Eight yaks, 5 by G-MeOH and 3 by SC-MeOH were completely cured. G-MeOH (83.33%: 5/6) exhibited the most prominent clinical efficacy followed by SC-MeOH (50%: 3/6).The effectiveness of extracts was noticed from the third day onwards. However, no clinical recovery was observed in the yaks with vehicle control up to 7 days. Garlic is active against wide range of gram positive and gram-negative bacteria even against antibiotic resistant strains (Ashan *et al.* 1996, Ankari and Mirelman 1999). However, the response may vary depending upon the bacterial species. The proposed active compound of garlic is allicin or di-allyl-thiosulphinic acid, which is released from a precursor when the garlic bulbs are crushed (Sasmal *et al.* 2005). The root of costus is commonly used as herb and Ayurvedic medicine where it is used mainly as tonic, stimulant and antiseptic (Yeung 1985, Tsarong 1994). The root is also used as anodyne, antibacterial, antispasmodic, aphrodisiac, carminative, skin stimulant, stomachic, tonic and vermifuge (Yeung 1985). The antimicrobial effect could be attributed to different kinds of flavonoids and sesquiterpene lactone derivatives like anthocyanins, ellagitannins, and proanthocyanidin, which were reported to be present in this plant.

This preliminary study is able to open the floodgate of new knowledge as the literature on the treatment of keratoconjunctivitis by some herbal compound is not available. It can be concluded from the results that G-MeOH and SC-MeOH supported the traditional wisdom of herbal remedy use and suggested a potential clinical testing by improving the product formulation with greater efficacy and duration of action would be of interest and awaits further investigation.

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REFERENCES

Ahsan M, Azad Chowdbury A K, Islam S N and Ahmed Z U. 1996. Garlic extract and allicin: Broad spectrum antibacterial agents effective against multiple drug resistant strains of *Shigella dysenteriae* type 1 and *Shigella flexneri,* Enterotoxigenic *Escherichia coli* and *Vibrio cholerae*. *Phytotherapy Research* **10**: 329–31.

Akerstedt J and Hofshagen M. 2004. Bacteriological investigation of *i*nfectious keratoconjunctivitis in Norwegian sheep. *Acta Veterinariae Scandinavia* **45**: 19–26.

Ankari S and Mirelman D. 1999. Antimicrobial properties of allicin from garlic. *Microbes Infection* **1**: 125–29.

Bandyopadhyay S, Saravanan BC, Ghosh M K, Sarkar M, Ramesha K P and Bhattacharya M. 2007a. *Common Health Ailments of Yaks* (*Poephagus grunniens* L.). Technical bulletin, NRC-Y publication, Dirang, AP.

Bandyopadhyay S, Ghosh M K and Rajkhowa J. 2007b. Unilateral trummatic keratoconjunctivitis with corneal opacity in a Dzomo-a case report. *Indian Veterinary Journal* **84**: 296–97.

Bandyopadhyay S and Bhattacharya M. 2008. Scope constraints and strategies of yak husbandry in poverty alleviation and development of hilly regions. Compendium of National Seminar on "*Integrated farming systems relevant to NE regions*". February 07–08, 2008, at College of *Veterinary* Science and Animal Husbandry, Central Agricultural University, Selesih, Aizwal, Mizoram, pp 82–85.

Bandyopadhyay S, Sasmal D, Ghosh M K, Borah B, Bora M and Biswas T K. 2008a. Collibacillosis in new born yak calves and antibiogram profile of rectal flora isolated from the same. *Indian Journal of Animal Sciences* **78**: 1101–02.

Bandyopadhyay S, Sasmal D, Dutta T K, Ghosh M K, Sarkar M, Sasmal N K and Bhattacharya M. 2008b. Seroprevalence of brucellosis in yaks (*Poephagus grunniens*) in India and evaluation of protective immunity to S19 vaccine. *Tropical Animal Health Production* (*in press*; DOI 10.1007/s11250–008–9228–0)

Bandyopadhyay S, Sasmal D, Ghosh M K, Bora M and Bhattacharyya M. 2008c. Antibacterial efficacy of neem (*Azadirachta indica*) against *Morexella bovis* and *Neisseria*. *Indian Journal of Animal Sciences* **78**: 1105–07.

Bandyopadhyay S, Sasmal D, Bora M, Ghosh M K, Sarkar M. 2008d. Antibacterial profile of *Rubus idaeus* (raspberry): An *in vitro* study. *Indian Journal of Animal Sciences* **78**: 1247–49.

Bandyopadhyay S, Chakrabarty D, Sarkar T, Pal B, Sasmal D, Biswas T K, Ghosh M K and Sarkar M. 2008e. A serological survey of bovine herpesvirus type –1 antibodies in yaks (*Poephagus grunniens*). *Review Scientific Technique Office International Epizootics* (*in communication*).

Bauer A W, Kirby W M M, Shemis J C and Truck M. 1966. Antibiotic susceptibility testing by a standard simple disc method. *American Journal of Clinical Pathology* **45**: 493–96.

Radostits O M, Gay C C, Blood D C and Hinchcliff K W. 2000. *Veterinary Medicine, A textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats.* 9th edn, WB Saunders Company Ltd.

Sasmal D, Babu Surendra C and Abraham T J. 2005. Effect of garlic (*Allium sativum*) extract on the growth and disease resistance of *Carrassius auratus* (Linnaeus, 1758). *Indian Journal of Fishes* **52**: 207–14.

Tsarong T J. 1994. *Tibetan Medicinal Plants*. Tibetan Medical Publications, India.

Yeung Him C. 1985. *Handbook of Chinese Herbs and Formulas.* Institute of Chinese Medicine, Los Angeles.

Williams J D, Moosdeen F, Teoh-Chan C H, Lim V K E and Jayanetra P. 1989. Surveillance of antibiotic resistance in South East Asia. *Euroepan Journal of Epidemiology* **5**: 207–13.