Research Article

EFFECTS OF ALCOHOLIC EXTRACTS OF *MALLOTUS PHILIPPINENSIS* (KAMALA) AND *HEDYCHIUM SPICATUM* (KAPOOR KACHRI) ON ISOMETRICALLY MOUNTED GASTROTHYLAX *CRUMENIFER*

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ABSTRACT

In view of unavailability of an effective vaccine and development of resistance against synthetic anthelmintics, plants have been considered as dependable source for the development of new anthelmintic agents. In the present investigation, effects of alcoholic extracts of *Mallotus philippinensis* (Kamala) and *Hedychium spicatum* (Kapoor Kachri), were evaluated on gross visual motility and spontaneous muscular activity of isometrically mounted *Gastrothylax crumenifer*. Alcoholic extract of *M. philippinensis* caused marked reduction in baseline tension and frequency of isometrically mounted amphistomes in a concentration-dependent manner. Gross visual motility of the amphistomes was completely inhibited by 3000 µg/ml concentration alcoholic extract of *M. philippinensis* and *H. spicatum*. In conclusion, the findings of the present study indicate that the alcoholic extract of *M. philippinensis* possesses strong anthelmintic activity against *G. crumenifer in vitro*.

Key words: *Gastrothylax crumenifer*, *Mallotus philippinensis*, *Hedychium spicatum*, isometric recording.

INTRODUCTION

Helminthosis is one of the most important animal diseases worldwide, inflicting heavy production losses in grazing animals. In a tropical country like India, the production losses are enormous because majority of the farm holdings are marginal or small scale ones. Amongst helminths, trematodes have both veterinary and public health significance. They are mainly liver flukes (*Fasciola*), blood flukes (schistosomes), and amphistomes. Besides fasciolosis, amphistomiosis in ruminants also causes considerable economic loss. In India, numerous outbreaks of acute amphistomiosis associated with high mortality among young sheep, goat, cattle and buffaloes have been recorded (Panda and Mishra, 1980). *Gastrothylax crumenifer* is one of the most common amphistomes in the Indian subcontinent (Prasad and Varma, 1999).

Chemotherapy is the only efficient and effective method to control helminthic infection, as efficacious vaccines could not be developed against these parasites as yet (Babayan *et al.,* 2012a and b). However, conventional (synthetic) anthelmintics have some serious disadvantages, such as non-availability in some developing countries, high cost factor, risk of inadvertent use leading to drug resistance, environmental pollution and food residues (Hammond *et al.,* 1997). Consequently, there is an urgent need to develop newer, selective and ecofriendly agents to control helminth infections.

In view of the above, development of plant-based anthelmintics offers an alternative that can overcome some of these problems and could be both sustainable and environmentally acceptable. A number of medicinal plants have been used to treat parasitic infections in man and animals (Chopra *et al.,* 1956; Githiori *et al.,* 2006; Gouws *et al.,* 2012). Few examples of herbs and herbal preparations that are used as anthelmintics are: extract of male fern, *Dryopteris felix* mas used against cestodes and trematodes, arecoline from *Areca catechu* against tapeworms of dogs and poultry, kamala obtained from fruits of *Mallotus philippinensis* against cestodes and round worms (Srivastava *et al.,* 1967).

In *in vivo* studies, the alcoholic extract of *Peganum hermala* has been found efficacious in experimentally induced fasciolosis in guinea pigs (Hajare *at el.,* 2012). Alcoholic extract of *M. philippinensis* caused complete paralysis of *Fasciola gigantica in vitro* (Kushwaha, 2004). Scientific evaluation of medicinal plants against amphistomes, particularly against *G. crumenifer* is limited. Hence, the present study was undertaken to assess the effects of some medicinal plant extracts on *G. crumenifer in vitro*.

MATERIAL AND METHODS

Experimental material

Collection of rumen amphistomes

Fresh, active and mature *G. crumenifer* were collected from the rumen of freshly slaughtered goats at local abattoir in warm (38±1°C) Hank's balanced salt solution (HBSS) in an insulated container. They were kept in the BOD incubator at 38±1°C until further use. The amphistomes (*G. crumenifer*) were identified before experimentation.

Composition of Hank's balanced salt solution (HBSS)

Sodium chloride (NaCl) - 137 mM (8.0 g), Potassium chloride (KCl) - 5.4 mM (402.6 mg), Disodium

hydrogen phosphate (Na_2HPO_4) – 0.3 mM (53.44 mg), Potassium dihydrogen phosphate (KH_2PO_4) – 0.4 mM (54.43 mg), Sodium hydrogen carbonate ($NaHCO_3$) – 4.2 mM (352.84 mg), Calcium chloride ($CaCl_2$) – 1.3 mM (191.12 mg), Magnesium chloride ($MgCl_2$) – 0.5 mM (101.65 mg), Magnesium sulphate ($MgSO_4$) – 0.6 mM (147.88 mg), D- glucose – 5.6 mM (1.0 g), Distilled water – 1000 ml, *p*H 7.4.

Collection of plant material

The plant materials were procured from the local market and were identified botanically before use. The pollen of seeds of *M. philippinensis* (Kamala) and rhizome of *Hedychium spicatum* (Kapur Kachri) were used in the study.

Preparation of alcoholic extract of the plants

The plant materials were dried under shade, powdered and then extracted with 70% ethanol under reflux. Ethanolic extract was concentrated under reduced pressure to a semi-solid mass.

Suspension/ Solution of drugs/ chemicals

The drug suspension (stock) was prepared as described below.

a) Oxyclozanide. A suspension of 10^{-2} M was prepared in Tween-80 (final concentration 0.1%) and distilled water and kept in a refrigerator at 4°C.

b) Plant extracts

Stock suspensions (100 mg/ml) of all the alcoholic extracts were prepared in Tween-80 (final concentration of Tween-80 was 0.1%) and distilled water and stored in refrigerator at 4°C. Dilutions of suspensions were prepared in HBSS just before their use.

Tissue preparation and mechanical recording of spontaneous muscular activity

The spontaneously active whole mature amphistome was mounted isometrically in HBS solution at 38±1°C as per the method earlier applied for *G. crumenifer* (Verma *et al.,* 2009a and b). In short, the amphistomes were mounted with the help of two fine hooks. One hook was inserted 1 - 2 mm caudal to anterior sucker and tied to the tip of aeration tube and another hook was pierced through the surface of acetabulum and connected to the isometric force transducer (Powerlab, AD Instruments, Australia).

The whole fluke was equilibrated without any tension for 30 min and then tension (200 mg) was applied. Control recordings were made for 15 min after application of tension and before administration of a drug/suspension. During the equilibration and control recording periods, the bath solution was changed once in every 10 min. Aeration is not required because amphistomes are anaerobic organisms.

Effect of cumulative concentrations of different medicinal plant extracts on the spontaneous muscular activity of G. crumenifer in vitro

Cumulative doses (100, 300, 1000 and 3000 µg/ ml) of the plant extracts were used on the isometrically suspended *G. crumenifer.* Each dose was allowed to act for 15 min and the spontaneous muscular activity of isometrically mounted amphistome was recorded in Chart Window 4 software programme.

Effects of plant extracts on the mass motility of G. crumenifer

To support the findings of *in vitro* motility studies, gross motility of G. *crumenifer* was studied in the presence of various concentrations of plant extracts. The mature, active amphistomes were incubated in the HBSS solution in Petri dishes containing normal HBSS, oxyclozanide (10^{-5}M) and different concentrations (100, 300, 1000 and 3000 µg/ml) of plant extracts for 4 h at 38±1°C. The gross motility of the worms were recorded visually 4 h after incubation and graded as 0, ±, +, ++, +++ and ++++, representing nil, feeble, poor, moderate, good and vigorous motility, respectively.

Statistical analysis

The results are presented as mean ± standard error of mean and to measure the level of significance, paired 't' test was applied (Snedecor and Cochran, 1989).

RESULTS

Recording of spontaneous muscular activity of G. crumenifer

The isometrically mounted amphistome exhibited the rhythmic contraction for hours. Three attributes, viz., amplitude, baseline tension and frequency of the rhythmicity were utilized to evaluate the effect of different concentrations of various drugs. The amplitude (average of all peaks per 10 minutes or average tension), baseline tension (average of all minimum levels of contractions used for measuring amplitude or average baseline tension) and frequency (total number of contractions in 10 min) of spontaneous muscular contractions recorded 15 min after applying the tension of 200 mg, were 0.72±0.03 g (n=6), 0.27±0.01 g (n=6), and 45±2.90 (n=6), respectively. The isometrically mounted amphistomes exhibited apparently uniform pattern of spontaneous muscular activity for a period of 2 h. There was no significant difference in amplitude (0.68±0.02 g; n=6), baseline tension (0.24±0.02 g; n=6) and frequency (43±3.44/10 min; n=6) of spontaneous contractions over a period of 2 h, as compared with those recorded 15 min after applying the tension to the amphistome.

Effect of plant extracts on the spontaneous muscular activity of G. crumenifer in vitro

Spontaneous muscular activity of *G. crumenifer in vitro* was studied in the presence of cumulative concentrations of different medicinal plant extracts and essential oils.

M. philippineneis

Table 1:
Effect of alcoholic extract of *M. philippinensis* on amplitude (g), baseline tension (g) and frequency (per 10 min) of spontaneous muscular activity of *G. crumenifer.*

Observations	Concentrations				
	Control	100 µg/ml	300 µg/ml	1000 µg/ml	3000 µg/ml
Amplitude (g)	0.72±0.09	0.52±0.07*	0.53±0.09*	0.50±0.05*	0.53±0.07*
Baseline tension (g)	0.43±0.04	0.29±0.02*	0.27±0.02*	0.15±0.01***	0.13±0.02***
Frequency/10 min.	46.00±1.99	42.33±1.87	30.00±1.54**	29.16±4.18**	25.30±5.25*

Table 2:
Effect of alcoholic extract of *H. spicatum* on amplitude (g), baseline tension (g) and frequency (per 10 min) of spontaneous muscular activity of *G. crumenifer.*

Observations	Concentrations				
	Control	100 µg/ml	300 µg/ml	1000 µg/ml	3000 µg/ml
Amplitude (g)	0.56±0.03	0.49±0.03	0.51±0.03	0.48±0.02	0.47±0.02
Baseline tension (g)	0.38±0.03	0.31±0.01	0.23±0.02*	0.21±0.01**	0.17±0.02**
Frequency/10 min.	47.40±1.9	45.60±2.26	48.20±3.21	51.73±3.52	55.38±3.19

Table 3:
Effect of oxyclozanide (10^{-5}M) and different concentrations of medicinal plant extracts (µg/ml) on gross motility of *G. crumenifer.*

Drug	Dose	Gross motility		
Control	–	+++		
Oxyclozanide	10^{-5} M	±		
	100	300	1000	3000
Mallotus philippinensis	++	±	±	0
Hedychium spicatum	++	+	+	+

Motility : 0 = nil, ± = feeble, + = poor, ++ = moderate, +++ = good

The extract of *M. philippinesis* produced inhibitory effect on rhythmic contractions of *G. crumenifer.* At 100 µg/ml and 3,000 µg/ml concentrations it decreased all the three attributes of rhythmic contractions of the flukes. The average baseline tension was reduced significantly (P<0.01) at 1000 µg/ml and 3000 µg/ml was 0.15±0.01 g and 0.13±0.02 g, respectively. The frequency was reduced significantly at 300, 1000 and 3000 µg/ml and was recorded 30±1.54, 29.16±4.18 and 25.3±5.25, respectively. The results are summarized in Table 1.

H. spicatum

Alcoholic extract of *H. spicatum* produced significant reduction in the baseline tension in concentration-dependent manner, as compared to control baseline tension (0.38±0.03g; n=6) of isometrically mounted amphistome. The baseline tension at 300, 1000 and 3000 µg/ml was 0.23±0.02, 0.21±0.01 and 0.27±0.02, respectively. The extract did not produce any significant changes in the frequency and amplitude of the rhythmic contractions. The results are shown in Table 2.

Effect of plant extract on gross motility of Gastrothylax crumenifer in vitro

The gross motility of *G. crumenifer* was observed by incubating the worms for 4 h in different concentrations of plant extracts and oxyclozanide. *G. crumenifer* incubated in HBSS without any drug was taken as control. Oxyclozanide (10^{-5} M) reduced the motility of worms to feeble (±). Motility of worms was nil in presence of *M. philippinensis* at 300 µg/ml concentration. Alcoholic extract *H. spicatum* reduced the motility of the amphistomes moderately (++) at higher concentrations of 1,000 µg/ml and 3000 µg/ml. The results are presented in Table. 3.

DISCUSSION

In view of the absence of efficacious vaccines against helminth parasites (Babayan *et al.,* 2012a and b), chemotherapy stands to be the major solution for these infections. But indiscriminate use of various anthelmintic agents enabled the helminths to develop resistance against these agents. Moreover, the synthetic agents produce residual toxicity to end users. Their inaccessibility in rural areas and high cost factor also necessitated greatly the need for development of anthelmintics from an alternative source i.e. plants. A myriad of botanical products have been in use since antiquity to treat or control various human or animals aliments in general and helminth parasites in particular (Chopra *et al.,* 1956; Githiori *et al.,* 2006; Gouws *et al.,* 2012).

The present study discusses the effect of various plant extracts on the spontaneous muscular activity of *G. crumenifer in vitro* in the presence of different concentrations of medicinal plants extracts are also studied. Different extracts were employed on the isometrically mounted *G. crumenifer* to evaluate the effect on its spontaneous muscular activity which is grossly similar to that of whole *S. mansoni* (Mellin *et al.*, 1983), *F. hepatica* (Fairwether *et al.*, 1983), *F. gigantica* (Kumar *et al.*, 1995) and *G. crumenifer* (Verma *et al.,* 2009a and b). The spontaneous muscular activity of the rumen fluke was

characterized by three attributes namely, frequency and amplitude of rhythmic contractions and the baseline tension. These attributes were compared before and following drug treatment as has been carried out for *F. hepatica* (Sukhdeo *et al.*, 1986).The changes produced by plant extracts in the *in vitro* motility studies were compared with gross visual motility (Kushwaha *et al.,* 2004; Hajare *et al.,* 2012).

The plants were selected on the basis of their reported anthelmintic activity against one or the other type of parasites. Oxyclozanide was taken as a reference drug in the present study. This drug is a salicylanilide, highly lipophilic and has high level of plasma protein binding. Oxyclozanide often cut by the uncoupling of oxidative phopsphorylation.

Apart from uncoupling of oxidative phosphorylation, oxyclozanide has been reported to inhibit acetylcholinesterase enzyme in flukes (Durrani, 1980; Renzik, 1988). In the present study, visual gross motility of *G. crumenifer* was feeble (±) in the presence of oxyclozanide (10^{-5}M).

Anthelmintic property of *M. philippinensis* has been reported against *F. gigantica* in *in vitro* studies (Kushwaha *et al.,* 2004). Alcoholic extract of *M. philippinensis* significantly reduced the frequency and amplitude of spontaneous muscular activity of the isometrically counted *G. crumenifer*. This observation was similar to the studies on *F. gigantica* by Kushwaha *et al.,* 2004. The average baseline tension of the isometrically mounted fluke was reduced considerably in a concentration-dependent manner. In gross motility studies, in the presence of alcoholic extract of *M. philippinensis* at concentration 300 and 1000 µg/ml, the motility was feeble (±). But at 3000 µg/ml the extract inhibited the gross motility of *G. crumenifer* completely which was graded as nil (0).

The significant reduction in the baseline tension of the isometrically mounted *G. crumenifer* suggests that the extract results in flaccid paralysis of the amphistome. However, the extract failed to completely paralyse the isometrically mounted amphistome.

Alcoholic extract of *H. spicatum* was found to be highly efficacious in experimentally induced fasciolosis in guinea pigs (Hajare, 2000). In the presence of various concentration of alcoholic extract of *H. spicatum*, the isometrically mounted *G. crumenifer* exhibited an increase in frequency in a concentration-dependent manner. Similar observation was reported in *in vitro* studies on *F. gigantica* (Hajare, 2000). Both amplitude and baseline tension of the spontaneous muscular activity of *G. crumenifer* was decreased by *H. spicatum* extract (1000 and 3000 µg/ ml). It is concluded that alcoholic extracts of *M. philippinensis* caused marked reduction in baseline tension and frequency of isometrically mounted rumen fluke in a concentration-dependent manner and *H. spicatum* reduced the gross visual motility of *G. crumenifer*.

REFERENCES

Babayan, S.A., Allen, J.E. and Taylor, D.W. (2012a). Future prospects and challenges of vaccines against filariasis. *Parasite Immunol.* **34**:243-253.

Babayan, S.A., Foster, N. and Elsheikha, H.M. (2012b). The immune response to parasitic helminths of veterinary importance and its potential manipulation for future vaccine control strategies. *Parasitol. Res.* **110**:1587-1599.

Chopra, R.N., Nayyar, S.L. and Chopra, I.C. (1956). Glossary of Indian Medicinal Plants. Council of Scientific and Industrial Research, New Delhi, p 160.

Durrani, M.S. (1980). The effect of some fasciolicides and organophosphate compounds on the activity of cholinesterase of *Fasciola hepatica* and *Fasciola gigantica*. *Pak. J. Zool.* **12**:85-92.

Fairweather, I., Holmes, S.D. and Threadgold, L.T. (1983). *Fasciola hepatica* a technique for monitoring *in vitro* activity. *Exp. Parasitol.* **56**:369-380.

Githiori, J.B., Athanasiadou, S. and Thamsborg, S.M. (2006). Use of plants in novel approaches for control of gastrointestinal helminths in livestock with emphasis on small ruminants. *Vet. Parasitol.* **139**:308-320.

Gouws C., Steyn, D., Du Plessis, L., Steenekamp, J. and Hamman, J.H. (2012). Combination therapy of Western drugs and herbal medicines: recent advances in understanding interactions involving metabolism and efflux. *Expert. Opin. Drug Metab. Toxicol.* **8**:973-984.

Hajare, S. (2000). Pharmacological and biochemical studies on some medicinal plants against *Fasciola gigantica*. PhD Thesis, Indian Veterinary Research Institute, Izatnagar.

Hajare, S.W., Lonare, M.K. and Kumar, D. (2012). Paralytic effect *Peganum harmala* Linn. Alcoholic extract on *Fasciola gigantica*. *J. Vet. Pharmacol. Toxicol.* **11**:53-56.

Hammond, J.A., Fielding, D. and Bishop, S.C. (1997). Prospects for plant anthelmintics in tropical veterinary medicine. *Vet. Res. Comm.* **21**:213-228.

Kumar, D., Mishra, S.K., Tandan, S.K. and Tripathi, H.C. (1995). Possible mechanism of anthelmintic action of palasonin on *Ascaridia galli. Indian. J. Pharmacol.* **27**:161-166.

Kushwaha, D.S., Kumar, D., Tripathi, H.C. and Tandan, S.K. (2004). Effect of some indigenous medicinal plant extracts on *Fasciola gigantica in vitro*. *Indian J. Anim. Sci.* **74**:17-20.

Mellin, T.N., Busch, R.D., Wang, C.C. and Kath, G. (1983).

Neuropharmacology of the parasitic trematode *Schistosoma mansoni*. *Am. J. Trop. Med. Hyg.* **32**:83-93.

Panda, B.K. and Mishra, S.C. (1980). Observations on the etiology, clinical pathology and chemotherapy of immature amphistomiasis in buffalo calves. *Indian J. Anim. Hlth.* **19**:131-135.

Prasad, A. and Varma, T.K. (1999). On the prevalence and community dominance among paramphistomes infecting domestic ruminants. *J. Vet. Parasitol.***13**:129-133.

Renzik, G.K. (1988). Anticholinesterase activity of anthelmintics on neuromuscular apparatus of *Fasciola hepatica*. *Veterinarya Moscow* **11**:43-43.

Snedecor, G.W. and Cochran, W.G. (1989) Statistical Methods ,6th Edn. Oxford and Indian Book House, New Delhi.

Srivastava, M.C., Singh, S.W. and Tiwari, J.P. (1967). Anthelmintic activity of *Mallotus philippinensis* powder. *Ind. J. Med. Res.* **55**:746-749.

Sukhdeo, S.C., Sangster, N.C. and Mettrick, D.F. (1986). Effects of cholinergic drugs on longitudinal muscle contraction of *Fasciola hepatica*. *J. Parasitol.* **72**:492-497.

Verma, P.K., Kumar, D., Tandan, S.K. (2009a). Functional role of cholinergic drugs on spontaneous muscular activity in the amphistome *Gastrothylax crumenifer* from ruminants. *J Helminthol.* **83**:77-82.

Verma, P.K., Kumar, D. and Tandan, S.K. (2009b). Effects of amino acid neurotransmitters on spontaneous muscular activity of the rumen amphistome, *Gastrothylax crumenifer*. *J Helminthol.* **83**:385-389.

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Research Article

ESTIMATION OF ANTIMICROBIAL DRUG USAGE FOR TREATMENT OF CLINICAL MASTITIS CASES IN ORGANIZED DAIRY FARM

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ABSTRACT

We estimated the incidence of clinical mastitis (CM) and antimicrobial agent (AMA) usage for its treatment in an organized dairy farm over a period of six months. The incidence of mastitis at different stages of lactation was also quantified. The incidence of mastitis was higher in Sahiwal cows (9.52%) compared to crossbred cows (3.61%). Among different AMA usage, enrofloxacin (22%), ampicilin with cloxacillin (18.57%), gentamicin (18.29%) and ceftriaxone (12.57%) drugs were most commonly used against CM cases. Penicillin group and their combinations (29.43%) was found to be the most commonly used for CM followed by fluoroquinolone (22%), aminoglycosides groups (21.43%) and cephalosporins group (16.86%) of drugs. Tetracyclines (9.14%) and chloramphenicol (1.14%) were least choice as evidenced by less frequent usage. From the findings of the study it may be concluded that the incidence of mastitis was high in Sahiwal cows milked with machine milking system and mastitis animals needed an average of five days treatment for clinical cure. Further, ?-lactam group of antimicrobial drugs were predominantly used in mastitis treatment, despite entry of new AMA in the market.

Key words: Clinical mastitis; Stage of lactation; Duration of treatment; Antimicrobial agent usage and organized dairy farm

INTRODUCTION

In modern dairy farming system, antimicrobial drugs are used for both therapeutic and prophylactic purposes. Most antimicrobial drugs are used for therapeutic purposes, while some are used for prophylactic purposes. Although antimicrobial drugs are used to treat various diseases of dairy cows, mastitis remains the most common cause of drug usage in dairy animals (Cha *et al.* 2011) and contributes to a substantial portion of veterinary costs incurred by the dairy industry. Further, mastitis was found to be one of the major reasons for antibiotic residue problem. The type of antimicrobial drugs used on dairy farms have been reported in USA (Zwald *et al.*, 2004; Kirk *et al.*, 2005, Sawant *et al.*, 2005). A Survey based study in Michigan, Minnesota, New York, and Wisconsin dairy farms found that usage of newer antibiotics such as ceftiofur and dry cow therapy was a common practice on these farms (Zwald *et al.*, 2004). European Medicines Agency (EMA) is carrying out the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project to establish national systems for the collection of data on sales of veterinary antimicrobial agents in Europe.

Understanding the type and magnitude of antimicrobial agent usage in dairy animals are important phenomenon and currently, no data available related to antimicrobial drug use in India. On other hand, these data are important to ensure the appropriate use of medically important antimicrobial agents in food-producing animals and for development of strategies to reduce the antimicrobial resistance which is critically important for protecting human and animal's health. For instances, the susceptibility profiles of recently obtained isolates appears to be most commonly resistance to the older drugs such as tetracyclines, streptomycin, penicillins, and sulfonamides while, resistance appears to be less prevalent, but emerging at varying rates, among the newer drugs like cephalosporins, quinolones and macrolides (Morley *et al.*, 2005). Although resistance of some bacteria to some drugs is prevalent, it is not uniform across all types of drugs and all bacterial species. Collectively it suggested that without epidemiologic pattern of antimicrobial drug usage over the period of time, it would be very difficult to correlate sensitivity pattern of organism. Recently, the food safety has become major issues due to public awareness and globalization. Among them, the veterinary drug residence in animal's products and antibiotic resistance in human and veterinary sector has become major area of concern. Antibiotics can enter the aquatic and terrestrial ecosystems through the discharge of farm effluents (Bates *et al.*, 1994). Further, when farm wastes are applied to the land, bioactive veterinary drug residues and antimicrobial-resistant bacteria are susceptible to runoff into water bodies and can create reservoirs for antibiotic resistant bacteria (Chee-sanford *et al.*, 2001). It is believed that extensive use of antibiotics is most important reason for bacterial resistance against antibiotics (Levy, 1992) through novel mutations or newly acquired characteristics to survive and proliferate (Baquero *et al.*, 1998). In order to develop guidelines like Maximum Permissible Level (MPL) or Maximum Residue Limits

(MRL), the quantification of the antimicrobial drug usage in the field conditions is important and foremost step for regulatory authorities. Since, such kind of data are not available in India, the objective of this study was to quantify antimicrobial drug usage in clinical mastitis which is believed to be top most reason for antimicrobial drug usage in dairy farms in world including India.

MATERIALS AND METHODS

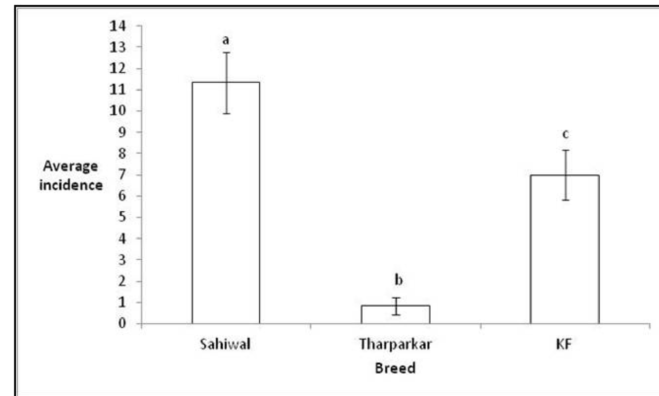
The present study was conducted at the Cattle Yard, National Dairy Research Institute, Karnal (Haryana) India. A subtropical climate prevails in the area. The experimental cows were maintained in loose housing system under group management. The cows were reared in paddocks with brick on edge flooring. The nutrient requirements of all experimental cows were mostly met with *ad-libitum* green fodder as per seasonal availability and measured amount of concentrate as per standard farm practices. Milking was done 3 times per day through milking machine.

Data on 119 clinical mastitis (CM) cases recorded over a period of six months was considered for the present study. The detection of clinical mastitis by Veterinarian was the routine procedure in the farm during milking time. Cows with one or more of the following clinical signs were considered as affected with CM: clotting of the foremilk, changes in color and consistency of the foremilk, hot, hard, or swollen udder. The treatment was given immediately after detection of CM and the animal was considered as clinically cured based on, absence of all the above mentioned clinical signs. Antimicrobial drugs usage and its duration were calculated from daily treatment register of the farm. To perceive the average number of treatment days required per CM cases, the treatment days were classified as short (2-3 days), long (4-8 days) and very long (> 8 days). To distinguish the effect of stage of lactation on incidence of CM the animals were divided into three stages of lactation (Early: 1 to 100 days; Mid: 100 to 200 days; Late: above 200 days). The incidence in indigenous (Sahiwal and Tharparkar) and crossbred (Karan Fires and Karan Swiss) cattle were recorded. The average milk production of indigenous cows was 6.9 and 5.58 Kg, respectively. The average number of crossbred milch animal during the study period was 194, and their average milk production was 11.5 and 10.63Kg, respectively.

RESULTS AND DISCUSSION

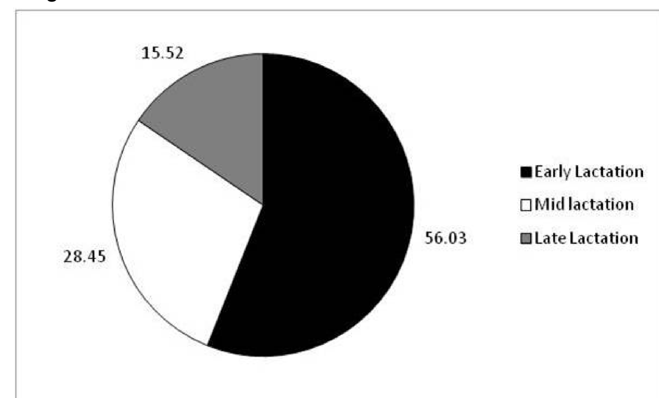
The incidence of average number of CM during the study period in indigenous and crossbred cows was 9.52 and 3.61% respectively (Fig. 1). The incidence was significantly ($p<0.001$) higher in Sahiwal as compared to Karan Fries cows. Our findings are contrary to the earlier reports (Sharma and Maiti, 2010) who reported high incidence of CM in crossbred cows compared indigenous

Fig 1:
Prevalence of clinical mastitis in different breeds in organized dairy farms.



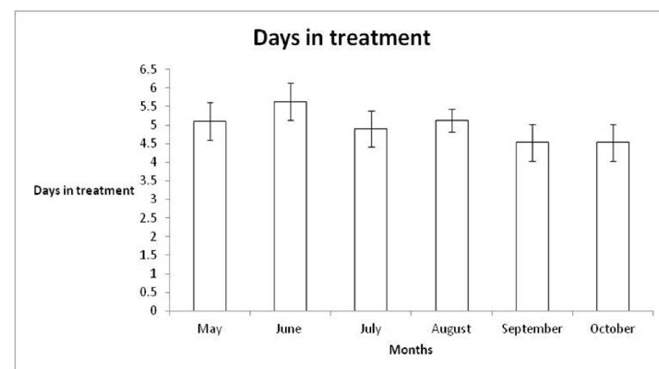
The values are average incidence of clinical mastitis per breed ($p<0.001$)

Fig 2:
Distribution of mastitis incidence (percentages) in different stages of lactation.



There was no significant difference between different stages of lactation in incidence (P=0.197)

Fig 3:
Effects of clinical mastitis on duration of treatment



The value are Mean ± SEM. Duration of treatment is not differing among different months (P=0.838)

cows. There is strong belief that indigenous cows of India are generally less affected by mastitis. Earlier studies were conducted in dairy farms where hand milking was practiced. However, in the present study all the animals were milked by machine milking. It is possible that machine milking practice has an impact on incidence of mastitis in indigenous cows, since the practice has not been well standardized with these animals. The less adaptability of Sahiwal cows to milking machine due to different anatomical and physiological characters of udder and teats compared to crossbred cattle could be the possible reason for the higher incidence. Therefore, while construction of milking unit, the anatomical features of zebu cattle need to be considered critically instead of blind adaptation of milking machine practices to Indian cattle. Effects of CM on different stage of lactations are presented in Fig 2. In the present study, percentage of CM was high in early lactation (56.03%) followed by mid (28.45%) and late (15.52%) lactation suggesting the risk of developing CM is high during early lactation (Corbett, 2009). Immunosuppression during peri-partum period along with alteration in energy metabolism and hormonal concentration could be the possible reasons for the higher incidence of CM during early lactation (Cai *et al.*, 1994).

Duration of the treatment is an important factor to decide success of therapy. To study the duration of treatment required to clinically cure the CM cases, we classified into short (2-3 days), long (4-8 days) and very long (>8 days) and found 64 % of the animals were subjected to long days treatment (4-8 days), followed 30% cows required as short term treatment (2-3 days) and six per cent of the animals required very long treatment in CM cases. The average number of days required for successful clinical cure in CM cases was found to be 5 days (Fig 3). Experimental and epidemiological studies have shown that longer duration treatment is generally associated with a higher probability of cure (Sol *et al.*, 2000; Deluyker *et al.*, 2005) in clinical and subclinical mastitis. It has been suggested that 2 to 3 days treatment is customary for clinical mastitis (Barkema *et al.*, 2006) and benefits of longer therapy like, higher cure rate, less risk of transmission, decreasing SCC and thus improved quality and price must be considered rather than drawbacks, such as the antibiotic cost, loss of milk due to withdrawal and residues problem (Gillespie *et al.*, 2002; Swinkels *et al.*, 2005). Very long treatment for small proportion (6%) animals might be due to chronic infections, repeated episodes of CM or due to non responsive organism such as fungus and *Candida spp*.

Usage of individual antimicrobial agent (AMA) (Table 1) in the present study shows that enrofloxacin (22%), ampicilin with cloxacillin (18.57%) and gentamicin (18.29) drugs were the most commonly used against CM cases followed by ceftriaxone (12.57%). Although, the quantification of individual AMA usage against CM cases was not reported in India, *in vitro* antibiotic sensitivity assay in CM milk samples has been reported by many workers and found highest sensitivity against above mentioned drugs (Kumar and Sharma, 2002; Dhakal *et al.*, 2007; Sumathi *et al.*, 2008; Hawari *et al.*, 2008; Junaidu *et al.,*

TABLE 1:
Usage of different types of antimicrobial agents against clinical mastitis in organized Dairy farm.

S.No.	Antibiotic class	No. of usage	%
1	Ceftriaxone	44	12.57
2	Ceftizoxime	6	1.71
3	Cefaperazone + Sulbactum	9	2.57
4	Ampicillin + Cloxacilin	65	18.57
5	Ampicilin + Sulbactum	11	3.14
6	Penicillin + Streptomycin	3	0.86
7	Amoxycillin + Dicloxacillin	6	1.71
8	Colistin sulfate + cloxacillin sodium	7	2
9	Enrofloxacin	77	22
10	Chloramphenicol	4	1.14
11	Oxytetracyciln	32	9.14
12	Gentamicin	64	18.29
13	Streptomycin	11	3.14
14	Benzathene penicillin	5	1.43
15	Fortified Procaine Penicillin	6	1.71
	Total	**350**	

TABLE 2:
Usage of different classes of antimicrobial agents against clinical mastitis in organized Dairy farm.

S.No.	Antibiotic class	No. of usage	%
1	Penicillin and their combinations	103	29.43
2	Cephalosporins group	59	16.86
3	Tetracycline group	32	9.14
4	Aminoglycosides group	75	21.43
5	Chloramphenicol group	4	1.14
6	Fluroquinolones groups	77	22
	Total	**350**	

TABLE 3:
Usage of different supportive therapy against clinical mastitis in organized dairy farm.

S. No	Supportive therapy	No. of usage	%
1	NSAIDs	59	34.1
2	Antihistamines	9	5.2
3	Immunostimulant	7	4.05
4	Vitamins, Minerals & Amino acids	26	15.03
5	Miscellaneous	72	41.62
	Total	**173**	

NSAIDs means Non-steroidal anti-inflammatory drugs (meloxicum, ketoprofen, Nimesulide and paracetamol); Antihistamine drug is Chlorpheniramine Maleate; immunostimulant drug is levamisole; Miscellaneous includes some commercial preparation of antifibrinolytics, herbal preparations, etc.

2011). Usage of different classes of AMA (Table 2) and supportive therapy (Table 3) against CM in this study shows that penicillin and/or their combinations are the most common drugs (29.43%) used against CM. Zwald *et al.* (2004) reported that penicillin was the most commonly used (42.5%) drug against mastitis in conventional dairy farms at Michigan, Minnesota, New York and Wisconsin area in USA. Other studies have also reported that ²-lactams drugs were most commonly used for treatment of mastitis (Sato *et al.*, 2005; Sawant *et al.*, 2005). Tertacyclines group and chloramphenocol were least choice among different class of AMA. Although the sensitivity assay have not examined in our study, poor sensitivity to chlora, mphenicol and oxytetracycline was reported recently by Peer and Bhattacharyya, (2008) in India. The supportive therapy like, NSAIDs, antihistamines and micronutrient preparations for CM cows have been used routinely (Shpigel *et al.*, 1994; Huxley, 2004). However, there were no data available regarding the magnitude of usage. Among supportive therapy, NSAIDs like Meloxicum, Ketoprofen, Nimesulide and Paracetamol were found to be predominantly used followed by antioxidant micronutrient preparations like vitamins A, E and minerals (Cu. Zn, etc.). The beneficial roles of vitamin E (Batra *et al.*, 1992) and meloxicum or ketoprofen were reported by Banting *et al.* (2000; 2008).

Despite entrant of many antibiotics in veterinary medicines, penicillin group of antibiotics are still the most common choice for clinical mastitis. However, the magnitude of usage of individual antibiotics is not according to their age of discovery suggesting a changing pattern with choice of veterinarians or drug sensitivity against the organism. Further studies are necessary in longer period at different agro-climatic zones for distribution of mastitis pathogens, its sensitivity against drugs along with antimicrobial usage in veterinary practice.

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REFERENCES

Banting, A., Schmidt, H. and Banting, S. (2000). Efficcy of meloxicum in lactating cows with E.cloi endotoxin-induced acute mastitis. *J. Vet. Pharmacol. Therap.* **23:** E4.

Banting, A., Banting, S., Heinonen, K. and Mustonen, K. (2008). Efficacy of oral and parenteral ketoprofen in lactating cows with E.coli endotoxin-induced acute mastitis. *Vet. Record.* **163:** 506-09.

Baquero, F. Negri, M.C., Morosini, M.I. and Blazquez, J. (1998). Antibiotic-selective environments. *Clin. Infect. Dis.* **27:** S5–S11.

Barkema, H. W., Schukken, Y. H. and Zadoks, R. N. (2006). *Invited Review:* The Role of Cow, Pathogen, and Treatment Regimen in the Therapeutic Success of Bovine *Staphylococcus aureus* Mastitis. *J. Dairy Sc..* **89:** 1877–95.

Bates J, Jordens J Z and Grifûths D T. (1994). Farm animals as a putative reservoir for vancomycin-resistant enterococcal infection in man. *Antimicrob. Agents and Chemotherapy.* **34:** 507–14.

Batra, T. R., Hidiroglou, M. and Smith, M.W. (1992). Effect of vitamin E on incidence of mastitis in dairy cattle. *Canadian J. Animal Sc.* **67:** 775-88.

Cai, T. Q., Weston, P. G., Lund, L. A, Brodie, B., McKenna, D. J. and Wagner, W. C. (1994). Association between neutrophil functions and periparturient disorders in cows. *American J. Vet. Res.* **55:** 934–43.

Cha, E., Bar, D., Hertl, J. A., Tauer, L. W., Bennett, G., Gonzalez, R. N., Schukken, Y. H., Welcome, F. L., Grohn, Y. T. (2011). The cost and management of different types of clinical mastitis in dairy cows estimated by dynamic programming. *J. Dairy Sc.* **94:** 4476-87.

Chee-Sanford, J. C., Aminov, R. I., Krapac, I. J., Garrigues-Jean- Jean, N. and Mackie, R.I. (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Apppl. Environ. Microbiol.* **67:** 1494–502.

Corbett, R. (2009). Minimizing the effects of immunosuppression through management and nutrition. *Proceedings of the 48th Annual Meeting of the NMC* Charlotte, NC. January 25-28, 2009. Pp.113-19.

Dhakal, I. P., Dhakal, P., Koshihara, T. and Nagahata, H.(2007). Epidemiological and bacteriological survey of buffalo mastitis in Nepal. *J. Vet. Med. Sc.* **69:** 1241–45.

Deluyker H A, Van Oye S N and Boucher J F. (2005). Factors affecting cure and somatic cell count after pirlimycin treatment of subclinical mastitis in lactating cows. *J. Dairy Sc.* **88:** 604–614.

Gillespie, B. E., Moorehead, H., Lunn, P., Dowlen, H. H., Johnson, D. L., Lamar, K. C., Lewis, M. J., Ivey, S. J., Hallberg, J. W., Chester, S. T. and Oliver, S. P. (2002). Efûcacy of extended pirlimycin hydrochloride therapy for treatment of environmental Streptococcus spp. and Staphylococcus aureus intramammary infections in lactating dairy cows. *Veterinary Therapeutics* **3:** 373–80.

Hawari, A. D. and Al-Dabbas, F. (2008). Prevalence and Distribution of Mastitis Pathogens and their Resistance against Antimicrobial Agents in Dairy

Cows in Jordan. *American Journal of Animal and Veterinary Sciences* **3:** 36-39.

Huxley, J. (2004). Clinical forum - E. coli Mastitis. *Cattle practice* **9:** 1-7.

Junaidu, A. U., Salihu, M. D., Tambuwala, F. M., Magaji, A. A. and Jaafaru, S. (2011). *Advances in applied science research* **2(2):** 290-94.

Kirk, J. H., McCowan, B., Atwill, E. R., Glenn, K. S., Higgin-botham, G. E., Collar, C. A., Castillo, A., Reed, B. A., Peterson, N. G. and Cullor, J. S. (2005). Association of minimum inhibitory concentration cluster patterns with dairy management practices for environmental bacteria isolated from bulk tank milk. *J.Dairy Sc.* **88:** 3710–20.

Kumar R and Sharma A. (2002). Prevalence, etiology and antibiogram of mastitis in cows and buffaloes in Hissar, Haryana. *Indian J. Animal Sc.* **72:** 361-63.

Levy S B. (1992). Active efflux mechanisms for antimicrobial resistance. *Antimicrob. Agents and Chemotherapy.* **36:** 695-703.

Morley, P. S., Apley M. D., Besser, T. E., Burney, D. P., Feforka-cray, P. J., Papich, M. G., Traub-Dargatz J L and Weese J S. (2005). *J. Vet. Internal Med.* **19:** 617-29.

Peer, F. U. and Bhattacharyya, H. K. (2008). Comparative efficacy of fluorquinolone verses chloramphenicol and oxytetracycline in bovine mastitis. *Indian J. Animal Res.* **42(3)**: 225-26.

Sato, K., Bartlett, P. C., Erskine, R. J. and Kaneene, J. B. (2005). A comparison of production and management between Wisconsin organic and conventional dairy herds. *Livestock Prod. Sc.* **93:** 105–15.

Sawant, A. A., Sordillo, L. M. and Jayarao, B. M. (2005). A survey on antibiotic usage in dairy herds in Pennsylvania. *J. Dairy Sc.* **88:** 2991–99.

Shpigel, N. Y., Chen, R., Winkler, M., Saran, A., Ziv, G. and Longo F. (1994). Antiinflammatory ketoprofen in the treatment of field cases of bovine mastitis. *Res. Vet. Sc.* **56:** 62-68.

Sol, J., Sampimon, O. C., Barkema, H. W. and Schukken, Y. H. (2000). Factors associated with cure after therapy of clinical mastitis caused by *Staphylococcus aureus*. *J. Dairy Sc.* **83:** 278–84.

Sumathi, B. R., Veeregowda, B. M. and Amitha, R. G. (2008). Prevalence and antibiogram profile of bacterial Isolates from clinical bovine mastitis. *Vet. World* **1(8):** 237-38.

Swinkels, J. M., Rooijendijk, R. G., Hogeveen, H. and Zadoks, R. N. (2005). Economic beneûts of lactational treatment of subclinical Staphylococcus aureus mastitis. *J. Dairy Sc.* **88:** 4273–87.

USDA APHIS:VS:CEAH. (2005). Part IV: Antimicrobial use on U.S. dairy operations, 2002. Online:http://www.aphis.usda.gov/vs/ceah/ncahs/nahms/dairy/index.htm#dairy02.Accessed Jan. 15, 2006.

Zwald, A. G., Ruegg, P. L., Kaneene, J. B., Warnick, L. D., Wells. S. J., Fossler, C. and Halbert, L. W. (2004). Management Practices and Reported Antimicrobial Usage on Conventional and Organic Dairy Farms. *J. Dairy Sc.* **87:** 191–201.

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Research Article

CYTOPROTECTIVE EFFICACY OF HYDROETHANOLIC EXTRACT OF *BACOPA MONNIERI* IN ETHANOL AND ACETAMINOPHEN EXPOSED HepG2 CELLS

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ABSTRACT

This study was done for evaluation of cytotoxicity and cytoprotective action of hydroethanolic extract of *Bacopa* monnieri(HEBM) using HepG2cell line.Cell cytotoxicity was determined by MTT based cell cytotoxicity assay. Acetaminophen and HEBM both alone and ethanol+acetaminophen in combination showed a concentration dependent decrease in cell viability. The IC_{50} was calculated as 56µg /ml for acetaminophen, 174µg/ml for HEBM and 50µg/ml +15µg/ ml for ethanol + acetaminophen. Hepatotoxicity marker enzymes mainly AST, ALT, ALP and GGT were estimated in cell lysate and total proteins were estimated in cell supernatant. For estimation of enzymes and total proteins, four groups were made where group I served as control, Group II as negative control, group III as positive control and group IV as extract treated group. The cells were exposed to ethanol and acetaminophen in a concentration of 50µg/ml +15µg/ml. The treatments with plant extract and silymarin were given in the concentration of 20µg/ml and 10µg/ml, respectively. ALT, AST, GGT and ALP levels were significantly ($p<0.05$) lowered down in Group II. A decrease in total protein in HepG2 cell supernatant was observed in ethanol and acetaminophen treated group. The total protein level was increased in extract treated group which was comparable to the values in silymarin treated group. Similarly, the altered enzyme levels were significantly restored towards control group on plant extract treatment. It is concluded from this study that *Bacopa monnieri* possessed cytoprotective activity against ethanol-acetaminophen exposed HepG2 cells.

Key words: Acetaminophen, Bacopa monnieri, cytotoprotective activity, hydroethanolic extract, HepG2 cells.

INTRODUCTION

Bacopa monnieri, commonly known as Brahmi, is a creeping herb belonging to the family S*crophulariaceae* and commonly grows in marshy areas throughout India in all plain districts ascending to an altitude of 1,320m. The plant has been used by Ayurvedic medicinal practitioners in India from ancient times (Chopra *et al., 1956; Aiyer and Kolemmal,1964)*. The plant is used as a drug to improve memory and intellect, as a cardiotonic, digestant and to improve respiratory function in cases of bronchoconstriction (Nadkarni, 1988). *Bacopa monnieri* is rich in many phytocompounds including alkaloids like brahmine and herpestine, saponins like d-mannitol, hersaponin, acid A and monnierin, flavonoids (luteolin and apigenin) and sterols responsible for its potent antioxidant activity.

HepG2 is a human hepatoblastoma cell line with a wide variety of signal responses to different kinds of drugs**.** HepG2 cell line is also a popular and an effective *in vitro* model for assessing hepatoprotective potential of phytocompounds due to its functional similarity with an intact liver and thus was used to evaluate the cytoprotective efficacy of HEBM in this study.

MATERIALS AND METHODS

Preparation of hydroethanolic extract by the method (Singh, 2008) and presence of phytochemical qualitatively analysed by method (Harborne, 1973; Sofawara, 1982).

Maintenance of HepG2 cell line

The cell line was procured from NCCS, Pune. The cells were grown in Minimum Essential Medium (MEM) supplemented with 10% foetal bovine serum and 5% CO_2.

MTT based cell cytotoxicity assay

The stock solutions of each drug were prepared and filter sterilized. HepG2 cells were grown in two different 96-wells plastic plates (Greiner, Germany) at $1x10^5$ cells/ wells in all the wells except the wells of 1st and 12th column. The plate was then incubated for 24 hrs in humidified atmosphere with 5% CO_2 at 37^0C temperature. After the growth period of 24 hrs, the media was changed in each plate and the cells were treated with increasing concentrations of acetaminophen as 5-100 µg/ml (5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100µg/ml), ethanol+acetaminophen as 40+5µg/ml and 50+15µg/ml and plant extract 5-600µg/ml (5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 100, 200, 300, 400, 500µg/ml) from 3rd -10th column and incubated for 24 hrs at 37°C in a CO_2 incubator. Each concentration was kept in triplicates. Subsequently, 20µl of MTT solution (5mg/ml) was added to each of the wells, and further incubated for 4 hrs. After incubation, dark blue formazan crystals were solubilized with 200µl of DMSO. Then, absorbance was measured at 570nm

TABLE 1:
Experimental design for enzyme estimation in HepG2 cells

S.No.	Groups	Treatment
I	Control	——
II	Ethanol and Acetaminophen	50 μg/ml +15 μg/ml
III	Ethanol and Acetaminophen+ Silymarin	50 μg/ml +15 μg/ml +10μg/ml
IV	Ethanol and Acetaminophen + HEBM	50 μg/ml +15 μg/ml +20μg/ml

TABLE 2:
Estimation of total protein and hepatic enzymes in HepG2 cell line following HEBM treatment in ethanol and acetaminophen exposed cells

		Parameters				
Groups	Treatment	AST(IU/L)	ALT(IU/L)	GGT(IU/L)	ALP(IU/L)	TOTAL PROT.(g/dl)
I	CONTROL	251.05±2.04	30.05±1.31	40.29±2.44	146.50±6.26	0.21±0.00
II	AAP+E	83.09±2.04[a]	1.76±1.02[a]	19.08±2.44	73.25±7.83[a]	0.13±0.01[a]
III	AAP+E+ SILYMARIN	109.61±0.00 [a,b] (15.78%)	26.52±2.04[b] (162.50%)	27.57±3.67 [a,b] (40.00%)	103.09±3.13 [a,b] (40.74%)	0.17±0.00 [a] (52.17%)
IV	AAP+E+HEBM	123.76±4.08[a,b,c] (5.26%)	47.73±3.06 [a,b,c] (87.50%)	29.69±2.44[a,b] (50.00%)	113.04±1.80 [a,b] (54.32%)	0.18±0.02 [a,b] (65.21%)

Note: Percent values inside parentheses in bold letters below the means of parameters of group V, VI, VII depict percentage protection given by treatments.
*(AAP=Acetaminophen; E=Ethanol and S=Silymarin)
Mean value bearing superscript a,b,c,d differ significantly ($p<0.05$) when compared within group in a column.
a= Significantly ($p<0.05$) different when compared with group I.
b= Significantly ($p<0.05$) different when compared to group II.
c= Significantly ($p<0.05$) different when compared to group III.
d= Significantly ($p<0.05$) different when compared to group IV.

(Freshney, 2000).

The percentage growth rate was calculated as per the formula:

$$\% \text{ Growth Rate} = \frac{(\text{Sample} - \text{Control})}{(\text{Normal} - \text{Control})} \times 100$$

Estimation of hepatic enzymes and total protein

The hepatic enzymes AST, ALT, ALP and GGT were estimated in cell lysate while total protein estimation was done in cell supernatant. As shown in Table 1, for the estimation of hepatic enzymes, each group (maintained in flasks in triplicates) except control group I was given treatment with ethanol+acetaminophen (50 μg/ml+15 μg/ml) and incubated for 24 hours. Later, group III was treated with silymarin (10μg/ml) and group IV was treated with plant extract (20μg/ml) and incubated further for 24 hours. After completion of 24 hours, cells were harvested in HEPES for preparation of buffer cell lysate. Later, cell lysate enzyme estimation was done.

For isolation of total protein in cell supernatant, media was poured off and cells were washed with DPBS. HEPES buffer was then added to each flask and cells were lysed by repeated pipetting. The homogenate was placed in a centrifuge tube at 4°C for 45 minutes and later centrifuged at 10,000rpm for 10 minutes. Supernatant was collected and 2mM of PMSF was added to it. The total proteins were estimated by the method of Bradford (1976).

RESULTS AND DISCUSSION

MTT based cytotoxicity assay

The cytotoxicity assay was performed for checking viability of HepG2 cells by exposing the cells with acetaminophen, ethanol-acetaminophen combination and HEBM. There was a dose dependent reduction in the cell viability when exposed to different concentrations of acetaminophen (Fig.1), ethanol-acetaminophen combination (Fig.2) and HEBM for 24 hrs (Fig.3). Acetaminophen within a concentration range of 5-100 μg/ml produced a dose dependent decrease in cell viability to nearly 2% at 100μg/ml. However, the HEBM showed dose-dependent reduction in percentage cell growth and showed 80% inhibition of cell growth at 600 μg/ml. The IC_{50} value was taken as the parameter of toxicity which was calculated to be approx. 56 μg/ml for acetaminophen alone, 50 μg/ml +15 μg/ml for ethanol + acetaminophen and 174 μg/ml for HEBM alone in the present study (Plates 1-4).

Being a potent toxicant, acetaminophen showed severe cytotoxicity than the extract. With the increase in concentrations of the drug and extract on HepG2 cell line, granulation, rounding and detachment of the cells were

Fig 1:
MTT based cell cytotoxicity assay after 24h exposure of HepG2 cell line to different concentration of acetaminophen

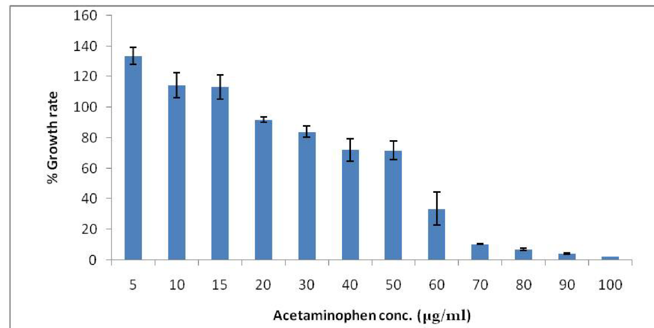


Fig. 2:
MTT based cell cytotoxicity assay after 24h exposure of HepG2 cell line to different concentration of ethanol and acetaminophen

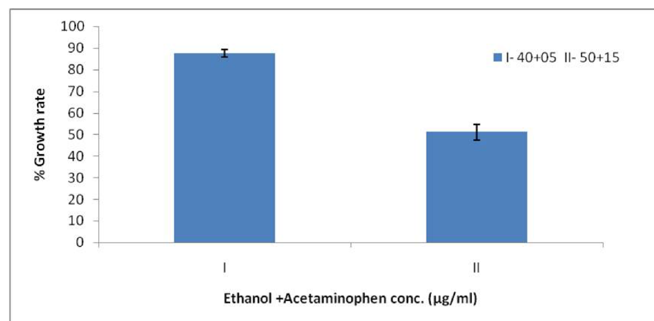


Fig 3:
MTT based cell cytotoxicity assay after 24h exposure of HepG2 cell line to different concentration of HEBM

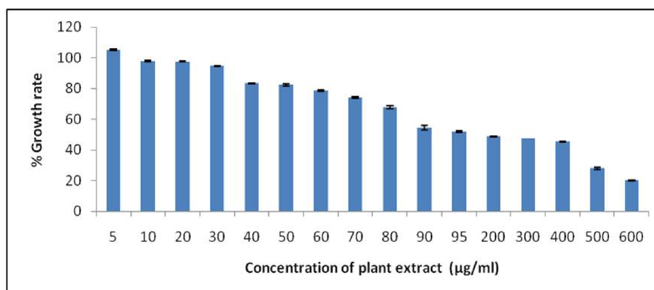


Plate 1:
Photomicrograph of HepG2 cell line showing normal epithelial like cells

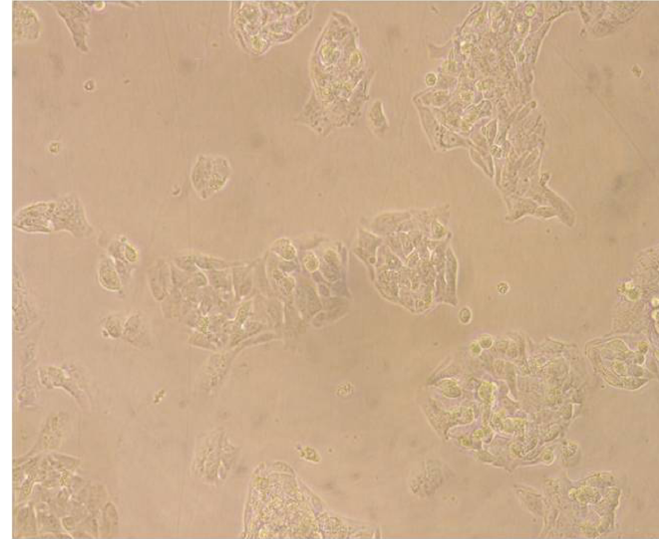
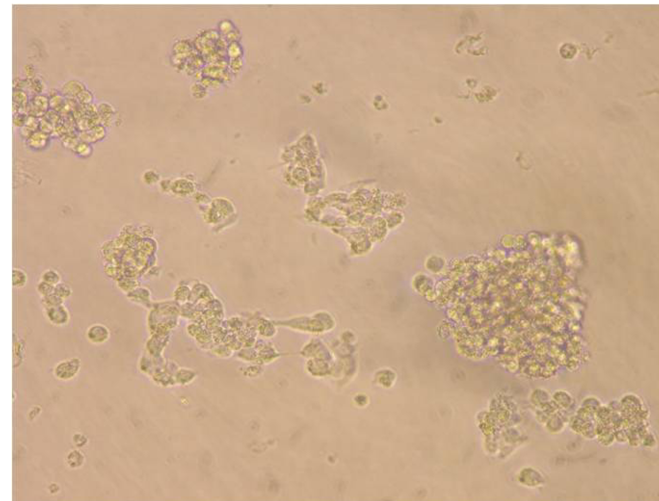


Plate 2:
Photomicrograph of HepG2 cell line showing toxic effect of acetaminophen at IC50 of 56μg/ml as clumping and rounding of cells along with degeneration of cell membrane



observed after 24 hours of incubation indicating cytotoxicity. Acetaminophen caused damage to the HepG2 cells in time and dose dependent manner. In cell cytotoxicity assay against ethanol-acetaminophen, IC_{50}, the dose at which 50 % cell viability is obtained, was determined, which was used for cytotoxicity in HepG2 cells for measurement of enzymes and total proteins.

Total protein and enzyme estimation

Much of the studies have been carried out by estimating enzyme in media. In our study, the enzymes were estimated in cell lysate. Hepatotoxicity is manifested by rise in serum levels of enzymes *in vivo* which is attributed to necrosis and loss of structural integrity of hepatocytes, resulting in release of the cytoplasmic enzymes into circulation after cellular damage. In *in vitro* studies, the serum is replaced by media. Thus, damage to HepG2 cells by ethanol and acetaminophen caused loss of structural integrity of the cells resulting in release of these enzymes in the media. In our study, as previously mentioned, the enzyme estimation was done in cell lysate, therefore, enzyme levels were reduced in negative control. The results of the enzyme profile can be correlated by total protein levels which were estimated in cell supernatant. The total protein levels were reduced in group II as compared to group I indicating cell damage.

Activity of ALT was significantly reduced in group II

Plate 3:

Photomicrograph of HepG2 cell line showing toxic effect of ethanol and acetaminophen at IC50 of 50+15µg/ml as clumping and rounding of cells along with degeneration of cell membrane

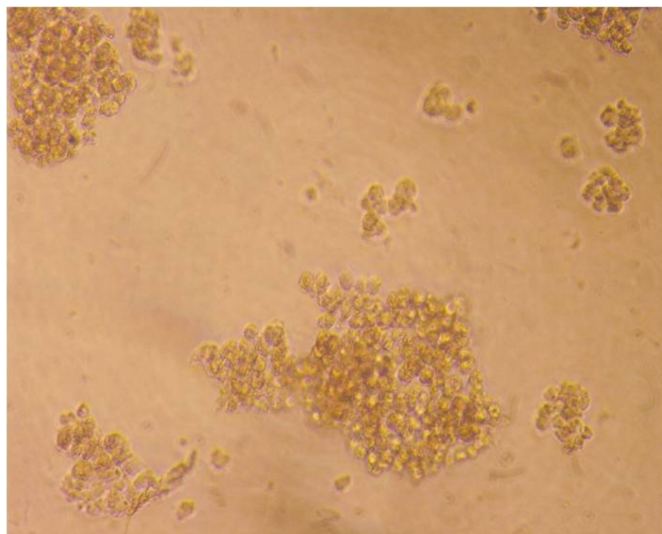
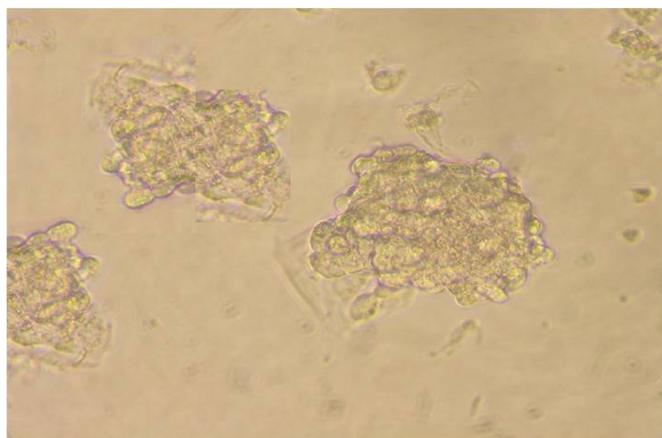


Plate 4:

Photomicrograph of HepG2 cell line showing toxic effect of HEBM at IC50 of 174µg/ml as clumping and rounding of cells along with degeneration of cell membrane



(1.76±1.02) when compared to group I (30.05±1.31). The enzyme activity was significantly ($p<0.05$) lower in treatment group than positive control but significantly ($p<0.05$) higher than control. The extract provided 87.50% protection in comparison to 162.50% by silymarin (Table 2).

Group II showed significantly ($p<0.05$) lowered AST levels (83.09±2.04) when compared to group I (251.05±2.04). Group III and IV showed significantly ($p<0.05$) higher AST levels when compared to group II. The enzyme activity was significantly ($p<0.05$) higher in extract treated group than silymarin. The extract had 5.26% protection against 15.78% as provided by silymarin (Table 2).

ALP levels were significantly ($p<0.05$) lowered in Group II (73.25±0.13) when compared to group I (146.50±0.11). Group III and IV showed significantly ($p<0.05$) higher ALP levels as compared to group I and II. 40.74% and 54.32% protection was provided by silymarin and extract, respectively, (Table 2).

Group II showed significantly ($p<0.05$) lowered GGT levels (19.08±2.44) when compared to group I (40.29±2.44). Group III and group IV showed significantly ($p<0.05$) higher GGT levels when compared to group II. The extract had 50% protection against 40% as provided by silymarin (Table 2). Since the enzyme levels were estimated in cell lysate, the values obtained wwere observed in an opposite fashion.

As shown in Table 2, the total protein levels were reduced significantly ($p<0.05$) in group II (0.13±0.01) when compared to control (0.21±0.00). There was a significant ($p<0.05$) increase in the total protein levels in group III and IV as compared to control. Group IV showed significant ($p<0.05$) rise in total protein levels when compared to group II. The extract had 65.21% protection against 52.17% as provided by silymarin.

It can be concluded from the study that the plant extract shows cytotoxicity at higher doses as compared to acetaminophen but has a protective role in ethanol+acetaminophen exposed HepG2 cell line.

REFERENCES

Aiyer, K.N. and Kolammal, M. 1964. Pharmacognosy of Ayurvedic drugs. Department of pharmacognosy, Trivendrum, University of Kerela. pp 27.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.

Chopra, R.N., Nayar, S.L. and Chopra, I.C. 1956. Glossary of Indian medicinal plants. Calcutta. pp 32.

Freshney, R.I. 2000. Culture Of Animal Cells: A Manual Of Basic Technique, 4th edn. John Wiey & Sons Inc. Publication, Newyork. 577p.

Harborne, J.B. 1973. Phytochemical methods. Chapman and Hall, London. pp 117.

Nadkarni, K.M. 1988. The Indian Materia Medica. South Asia Books, Columbia. pp. 624-625.

Singh, K.P. 2008. Elucidation of antioxidative and hepatoprotective properties of *Emblica officinalis* against mercury induced toxicity in rats and $HepG_2$ cell line. Thesis, Ph.D., G.B. Pant University of Agriculture and Technology, Pantnagar, p 40.

Sofawara, A. 1982. Medicinal plants and traditional medicine in Africa. Chichester, John Wiley.

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