Antagonistic potential of indigenous bacterial probiotics of Western Himalayas against antibiotic-resistant bacterial pathogens

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Eleven potential bacterial probiotic isolates of *Enterococcus*, *Bacillus* and *Lactobacillus* spp. were examined for their antagonistic activity against standard antibiotic-resistant pathogenic bacteria. Antagonistic activity was exhibited by most of these indigenous isolates against selected pathogens, but it was very strong against *Listeria monocytogenes*-MTCC 839 and *Staphylococcus aureus*-MTCC 96. These antagonistic substances were proteinaceous in nature, heat stable and retained their activity in presence of catalase, protease inhibitor and detergents. Based on these traits, the antagonistic substances were designated as bacteriocin-like substances. Hydrogen peroxide production was exhibited by only three isolates (two *Enterococcus faecium* and one *Lactobacillus fermentum*).

Keywords: Antagonistic activity, bacteriocin, hydrogen peroxide, probiotic, Western Himalayas.

LACTIC acid bacteria (LAB) are traditionally used as natural or selected starters in the preparation of various fermented foods. As they are involved in numerous food fermentations, it is assumed that most of the representatives of this group do not pose any health risk to man and are designated as generally recognized as safe (GRAS) organisms. The LAB, generally considered as 'food grade' organisms are mostly acclaimed as key member of probiotics and have been associated with various health-promoting traits. These LAB produce various antagonistic substances which can inhibit pathogenic and spoilage microorganisms[1]. Among all these antagonistic substances, bacteriocin production is often proposed as a beneficial characteristic of probiotic bacteria[2,3], which may facilitate the establishment of a probiotic strain in the competitive environment of the gut[2] and also contribute towards biopreservation of food product[4]. In Western Himalayas, traditional fermented foods are regularly being consumed by the people, since ages[5]. These fermented foods have been studied with respect to probiotic diversity[6]. However, they are yet to be evaluated with respect to the production of antagonistic substances against certain antibiotic-resistant bacterial pathogens to further confirm their utility as probiotics.

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A total of 102 bacteria were isolated from various traditional fermented food products (bhaturu, chilra, Lugri, fermented milk, seera, butter, cheese, Jan chang) of tribal areas of Western Himalayas by serial dilution technique on Man Rogosa Sharpe (MRS) agar medium. Of these bacterial isolates, on the basis of exhibition of various *in vitro* probiotic attributes such as acid-bile tolerance, tolerance to simulated gastrointestinal juices, hydrophobicity, etc., 11 potential bacterial probiotics, viz. *Enterococcus faecium* (AdF1 – GU396270; AdF2 – GU396271; AdF3 – GU396272 and AdF11 – GU396279), *Bacillus coagulans* (AdF4 – GU396273), *Lactobacillus plantarum* (AdF5 – GU396274; AdF6 – GU396275 and AdF10 – GU396278) and *Lactobacillus fermentum* (AdF7 – HQ677597; AdF8 – GU396276 and AdF9 – GU396277) were selected and characterized using molecular techniques[6]. For molecular characterization, the genomic DNA of 11 potential probiotic isolates was amplified by using genus-specific primers for *Enterococcus*, *Lactobacillus* and *Lactococcus lactis* subsp. *lactis* and species-specific primers for *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Lactobacillus fermentum*. Additionally, sequencing of the PCR products obtained through amplification with universal primers targeting 16S ribosomal gene of bacteria was also done to further confirm the primer-specific results. The details of primers[7–11] used for identifying indigenous isolates have been listed in Table 1. The sequences of bacterial isolates obtained after sequencing were blasted using on-line NCBI Blastn program http://www.ncbi.nih.gov/blast and submitted to GenBank, NCBI.

Six antibiotic-resistant indicator pathogenic bacteria, viz. *Listeria monocytogenes*-MTCC 839, *Pseudomonas aeruginosa*-MTCC 741, *Staphylococcus aureus*-MTCC 96, *Bacillus cereus*-MTCC 1272, *Shigella flexneri*-MTCC 1457 and *Escherichia coli*-MTCC 443 along with standard strains of *Lactobacillus plantarum*-MTCC 1407 and *Lactococcus lactis* subsp. *lactis*-MTCC 3041 (bacteriocin producing strain) were procured from the Institute of Microbial Technology, Chandigarh, India and were used in the antagonistic studies. The antibiotic resistance profile of these indicator pathogenic bacteria is depicted in Table 2.

The first screening of antagonistic activity was done by bit agar method[12] and second screening was carried out by triple-agar-layer method[13]. In both the methods inhibition zones produced around bit/colonies were recorded. The third screening was performed by well diffusion method[14,15]. In this method, the actions of lactic acid and hydrogen peroxide (H_2O_2) on test organisms were nullified by adjusting the pH of supernatant to 6.0 and by treating it with catalase (1 mg/ml final concentration) (catalase, EC 1.11.1.6, from bovine liver, Merck Specialities Pvt Ltd, Mumbai, India) respectively. These supernatants were heat treated for 5 min to inactivate the

Table 1. Primers used for identification of indigenous bacterial probiotics

Target	Primer	Primer sequence (5′–3′)	Reference
Lactobacillus genus	LbLMA1-rev	5′-CTC AAA ACT AAA CAA AGT TTC-3′	Dubernet *et al.*[7]
	R16-1	5′-CTT GTA CAC ACC GCC CGT CA-3′	
Enterococcus genus	Ent1	5′-TACTGACAAACCATTCATGATG-3′	Ke *et al.*[8]
	Ent2	5′-AACTTCGTCACCAACGCGAAC-3′	
Lactobacillus rhamnosus	Lu 5	5′- CTA GCG GGT GCG ACT TTG TT-3′	Unpublished
	Rha 11	5′- GCG ATG CGA ATT TCT ATT ATT-3′	
Lactobacillus plantarum	planF	5′-CCG TTT ATG CGG AAC ACC TA-3′	Torriani *et al.*[9]
	pREV	5′-TCG GGA TTA CCA AAC ATC AC-3′	
Lactobacillus fermentum	FERM1	5′-GTT GTT CGC ATG AAC AAC GCT TAA-3′	Chagnaud *et al.*[10]
	LOWLAC	5′-CGA CGA CCA TGA ACC ACC TGT-3′	
16S rRNA gene	16F	5′-AGA GTT TGA TCC TGG CTC AG-3′	Scola and Raoult[11]
	16R	5′-ACG GCT ACC TTG TTA CGA CTT-3′	

Table 2. Antibiotic resistance profile of indicator pathogenic bacteria used in the study

Pathogenic strains/antibiotics	Cn	Cd	Mt	Co	C	Nf	E	Cb	Cq	Fu	T	Nx	Of	Am	Cf	Na	P	G
Listeria monocytogenes-MTCC 839	S[a]	R[b]	R	S	R	S	S	S	R	R	S	R	S	S	R	R	R	R
Bacillus cereus-MTCC 1272	S	S	R	S	R	S	R	R	S	R	S	S	S	S	R	S	R	S
Pseudomonas aeruginosa-MTCC 741	R	R	R	R	R	R	R	S	R	R	R	S	S	R	S	R	R	R
Escherichia coli-MTCC 443	S	R	R	R	S	R	R	R	R	S	S	S	S	R	S	R	R	R
Staphylococcus aureus-MTCC 96	S	R	R	S	S	S	R	S	S	R	S	R	R	S	S	R	S	S
Shigella flexneri-MTCC 1457	S	S	R	R	S	R	S	S	R	R	S	S	S	R	S	R	R	S

Cn, Cefoxitin (30 μg/disc); Cd, Clindamycin (2 μg/disc); Mt, Metronidazole (5 μg/disc); Co, Co-trimoxazole (25 μg/disc); C, Chloramphenicol (30 μg/disc); Nf, Nitrofurantoin (300 μg/disc); E, Erythromycin (15 μg/disc); Cb, Carbenicillin (100 μg/disc); Cq, Cephadroxil (30 μg/disc); Fu, Fluconazole (10 μg/disc); T, Tetracyclin (30 μg/disc); Nx, Norfloxacin (10 μg/disc); Of, Ofloxacin (5 μg/disc); Am, Amoxycillin (10 μg/disc); Cf, Ciprofloxacin (5 μg/disc); Na, Nalidixic acid (30 μg/disc); P, Pencillin G (10 units/disc); G, Gentamycin (10 μg/disc). [a]S, Sensitive; [b]R, Resistant.

enzyme, centrifuged at 10,000 *g* for 10 min at 4°C and were then filter sterilized by using 0.22 μm Millipore filter membrane (Millex GV filter unit). The antimicrobial activity was quantified in terms of activity units (AU). One AU was defined as the reciprocal of highest dilution of supernatant showing a clear zone of inhibition of the indicator pathogen. Protein concentration in the supernatant was estimated by using standard Lowry method[16] with bovine serum albumin (BSA) as a standard. The antagonistic activity was expressed as activity units per μg of protein (AU/μg protein).

Antagonistic activity-possessing isolates were cultured in MRS broth for 16 h at 37°C. The cells were harvested (8000 *g*, 10 min, 4°C), the cell-free supernatant was adjusted to pH 5.0 with 1 M NaOH, heat-treated (80°C for 10 min) and precipitated with 80% saturated ammonium sulphate[17]. The precipitates were resuspended in 20 ml of 25 mM ammonium-acetate (pH 6.5) and the amount of antagonistic activity was determined by using indicator pathogenic bacteria as described above. In parallel, the antagonistic activity of the cell-free supernatant (adjusted to pH 5.0) of isolates was also determined before heating at 80°C for 10 min. The thermostability of the precipitated antagonistic substance was checked by heating it for different periods at temperatures ranging from 20°C to 121°C, and further testing their antagonistic activity by using indicator pathogenic bacteria.

The antagonistic substance precipitated was treated with trypsin (CDH Pvt Ltd, Mumbai), pepsin (Merck Specialities Pvt Ltd, Mumbai), proteinase K (Merck) and catalase (Merck) at a final concentration of 1 mg/ml of each enzyme. These enzyme solutions were prepared in 20 mM phosphate buffer (pH 7.0) and filter sterilized using 0.22 μm Millipore filter membrane (Millex GV filter unit). The enzyme-treated solutions were incubated at 37°C for 2 h. After this the enzymes were inactivated by heating at 80°C in water bath for 10 min and antagonistic activity was determined by using indicator pathogenic bacteria.

The effect of various chemicals like Triton X 100 (Merck), Tween 20 (Merck), Tween 80 (Merck), sodium dodecyl sulphate (SDS, Sigma, USA) and ethylene diamine tetraacetic acid (EDTA, a protease inhibitor, Merck) on the antagonistic activity was detected in a similar manner as described above for enzymes, but the final concentration of each chemical was 10 mg/ml (w/v or

v/v), and the tubes were incubated at 30°C for 30 min. After this the antagonistic activity was again checked against all the pathogens.

All indigenous bacterial probiotic isolates were screened for H_2O_2 production by qualitative method[18]. Isolates were spotted on a 20 ml MRS agar plate containing 5 mg of 3,3′,5,5′-tetramethylbenzidine (TMB; Merck), a benzidine-like chromogenic substrate of peroxidase and 0.20 mg of horseradish peroxidase (Peroxidase from Horseradish, EC 1.11.1.7, Merck) and incubated anaerobically for 48 h at 37°C. After incubation, the plates were kept open in the air for 10–15 min. Peroxidase generates O_2 from H_2O_2 produced by the organism and the TMB stains the colonies blue in the presence of O_2. The colonies that produced H_2O_2 on MRS agar would appear blue whereas non producers would be colourless.

The antagonistic activity was tested by three techniques, i.e. agar bit method, triple agar overlay method and well diffusion method to authenticate the antimicrobial properties of probiotics. By bit agar method, except isolates AdF6, AdF7 and AdF8, all the isolates exhibited antagonistic activity against *Listeria monocytogenes*-MTCC 839 and *Staphylococcus aureus*-MTCC 96. Isolates AdF1 to AdF6 and standard strain of *Lactococcus lactis* subsp. *lactis*-MTCC 3041 showed maximum inhibition zones (> 10 mm) against *Staphylococcus aureus* (Figure 1). Except isolates AdF7 and AdF10, all other indigenous tested bacteria showed antagonistic activity against *Staphylococcus aureus*-MTCC 96, *Bacillus cereus*-MTCC 1272 and *Listeria monocytogenes*-MTCC 839 with triple-agar-layer method. Only five isolates, viz. AdF1, AdF2, AdF3, AdF4 and AdF11 showed inhibitory activity against *Escherichia coli*-MTCC 443. Table 3 shows the results of third screening for antagonistic activity by well diffusion method. Isolate AdF1 showed the highest zone of inhibition (16 mm) against *Listeria monocytogenes*-MTCC 839 and also inhibited all the tested indicator pathogenic bacteria (Figure 2). Isolate AdF10, which did not exhibit any inhibition against *Listeria monocytogenes*-MTCC 839 in the triple layer agar overlay method, exhibited it in well diffusion method. When the results of antagonistic activity obtained with three types of techniques were examined, in general, the depiction of inhibitory activity was more with well diffusion assay in comparison to agar methods, probably due to the direct contact of antagonistic substance with indicator organism. Several other studies have reported this observation that the marked antimicrobial activity on agar media can be low for bacterial strains isolated from food sources[19,20]. The antagonistic substances produced by the indigenous isolates were extracellular and diffusible as inhibitory activity was also noticed in triple agar overlay method.

Lactobacillus plantarum-MTCC 1407 was inhibited by isolates AdF4 and AdF10. Interestingly, isolate AdF4 (*Bacillus coagulans*) was sensitive towards the antimicrobial substance produced by isolate AdF7 (*Lactobacillus fermentum*), which suggested that these isolates cannot be used in conjunction with each other. It is well established that certain LAB, in addition to their probiotic traits, also exhibit antagonistic activity against closely related bacterial strains[21,22] but here it was noticed across the genus. No inhibition was exhibited against one another by the rest of the bacterial probiotic isolates. Each indigenous strain exhibited a variable titre and pattern of inhibition against selected pathogens (Table 4) indicating thereby, that the inhibitory doses of the same antagonistic substance were different for different pathogens. This fact is also strengthened by the observations made by Annuk *et al.*[23] on *Lactobacillus* strains isolated from human infant faecal samples.

The antagonistic substance produced by indigenous bacterial probiotic isolates exhibited thermostability over a wide range of temperature. All the indigenous isolates except isolate AdF11 and standard bacteriocin producer strain of *Lactococcus lactis* subsp. *lactis* MTCC 3041 lost their antagonistic activity against *Listeria monocytogenes* after heat treatment at 70°C for 30 min. Isolate AdF10 could retain its antagonistic activity at 80°C for 15 min only. However, on autoclaving (121°C for 20 min), the antagonistic activity exhibited by AdF4, AdF10, AdF11 and standard bacteriocin producer strain was lost, whereas the activity of antagonistic substances produced by the rest of the indigenous bacterial probiotic isolates was retained even at this temperature. Various workers have also reported the production of heat stable antimicrobial substances by lactic acid bacterial strains such as *Lactobacillus*, *Lactococcus* spp. and *Enterococcus* spp. which have been characterized as bacteriocin(s)[17,24–26]. There was no loss of inhibitory activity after treatment with catalase, Tween 20, Tween 80, SDS and EDTA. After treatment with Triton X-100, loss of inhibitory activity of the antagonistic substance was observed, except for isolate AdF5, where the activity was retained but at reduced level (< 5 mm zone of inhibition). In some other studies, antimicrobial substances produced by *Lactobacillus* spp. and *Enterococcus* spp. were reported to lose their activity after treatment with SDS and Triton X-100 respectively[25,27]. With trypsin treatment, the antagonistic activity was completely lost in all the isolates except AdF9, AdF10 and AdF11. However, the antagonistic substances isolated from all indigenous isolates showed complete loss of their activity on treatment with pepsin and proteinase K. These observations confirmed their proteinaceous nature, and thus, allowed them to be considered as bacteriocins. On the basis of two important observations, i.e. proteinaceous nature of antagonistic substances and their heat stability, they can be considered as bacteriocins according to the criteria laid down by Tagg *et al.*[28] and Jack *et al.*[29]. Further, in view of their strong anti-listerial activity, they can tentatively be clubbed under class IIa bacteriocins[30,31]. As these substances have not been

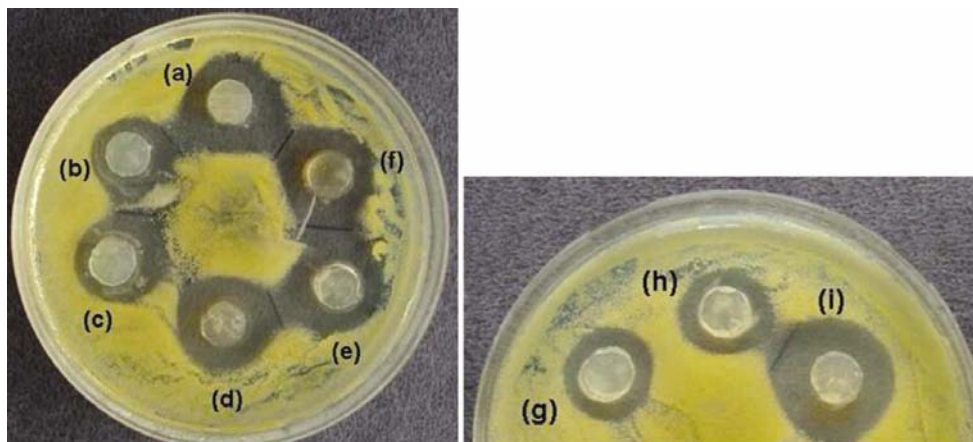

Figure 1. Antagonistic activity of isolate (a) AdF1; (b) AdF2; (c) AdF3; (d) AdF4; (e) AdF5; (f) AdF6; (g) AdF7; (h) AdF8; (i) *Lactococcus lactis* subsp. *lactis* (MTCC 3041) against *Staphylococcus aureus*-MTCC 96, as an indicator pathogenic bacteria.

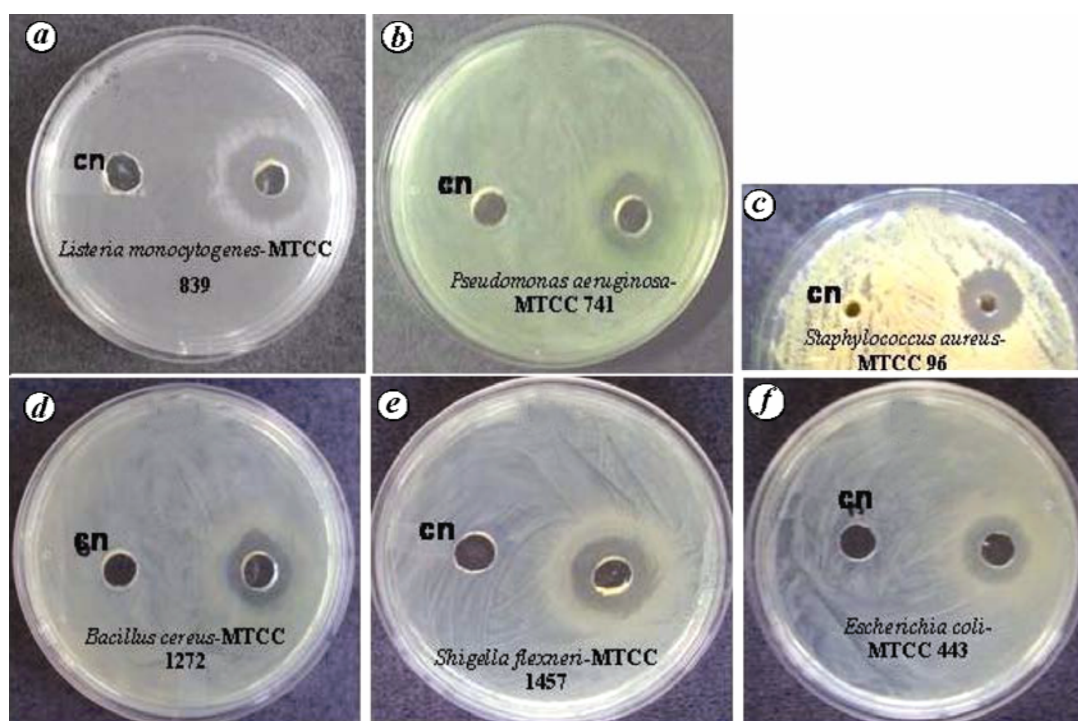

Figure 2. Antagonistic activity of isolate AdF1 (*Enterococcus faecium*) against (***a***) *Listeria monocytogenes*-MTCC 839, (***b***) *Pseudomonas aeruginosa*-MTCC 741, (***c***) *Staphylococcus aureus*-MTCC 96, (***d***) *Bacillus cereus*-MTCC 1272, (***e***) *Shigella flexneri*-MTCC 1457, and (***f***) *Escherichia coli*-MTCC 443. cn: control.

purified and characterized for amino acid and encoding nucleotide sequences, that would fully confirm their bacteriocin status, they are being designated as bacteriocin-like inhibitory substances (BLIS) as suggested by oglu Gulahmadov *et al.*[22]. This is the first report on BLIS production by indigenous bacterial probiotics of Western Himalayas. Further work on molecular analysis (characterization on the basis of molecular weight, amino acid and encoding nucleotide sequences) and purification of these BLIS is in progress in our laboratory.

Only three isolates, viz. AdF1, AdF2 and AdF7 were found positive for production of H_2O_2. *In vitro* H_2O_2 production is generally encountered in protective strains of genus *Lactobacillus* isolated from vagina and faecal samples[32]. This trait is rarely reported in probiotic strains isolated from fermented foods. Thus, the potential indigenous bacterial probiotic isolates possessing this trait may find their role in providing health benefits like improvement in vaginal health and/or urinary tract infections.

Table 3. Screening for antagonistic activity of indigenous bacterial probiotic isolates by well diffusion method

Isolate	*Listeria monocytogenes-* MTCC 839	*Pseudomonas aeruginosa-* MTCC 741	*Lactobacillus plantarum-* MTCC1407	*Staphylococcus aureus-* MTCC 96	*Bacillus cereus-* MTCC 1272	*Shigella flexneri-* MTCC 1457	*Escherichia coli-* MTCC 443
AdF1	++++[b]	++	–[a]	+++	++	+++	++
AdF2	++	+[e]	–	++	++	+	++
AdF3	+++[c]	++	–	++	+++	++	++
AdF4	+++	++	+	++++	+++	+	++
AdF5	+++	+	–	++++	+	+	+
AdF6	+++	+	–	+++	+	–	–
AdF7	–	–	–	++++	+	+++	–
AdF8	++[d]	++	–	++++	++	++	–
AdF9	+++	++	–	++++	+	+++	–
AdF10	+	++	+	++	++	–	–
AdF11	++	++	–	++++	+	++	++
MTCC 3041	++	–	–	+++	+	++	–

[a]–, No inhibition; [b]++++, More than 15 mm; [c]+++, 10–15 mm; [d]++, 5–10 mm; [e]+, 5 or <5 mm zones of inhibition.

Table 4. Quantification of antagonistic substances (in terms of activity units/μg protein) produced by indigenous bacterial probiotics

Isolate	*Listeria monocytogenes-* MTCC 839	*Pseudomonas aeruginosa-* MTCC 741	*Staphylococcus aureus-* MTCC 96	*Bacillus cereus-* MTCC 1272	*Shigella flexneri-* MTCC 1457	*Escherichia coli-* MTCC 443
AdF1	159.94	31.99	119.95	39.98	66.64	31.99
AdF2	142.52	97.80	47.51	38.00	63.34	118.76
AdF3	78.28	117.42	136.99	23.48	39.14	39.14
AdF4	78.00	34.32	156.00	15.60	72.80	7.80
AdF5	41.58	4.16	8.32	10.40	4.16	4.16
AdF6	83.20	3.33	83.20	13.31	–	–
AdF7	–[a]	–	193.70	48.43	32.28	–
AdF8	85.26	21.92	73.08	19.49	48.72	–
AdF9	71.26	38.00	142.52	19.00	95.81	–
AdF10	209.97	41.99	157.49	52.49	–	–
AdF11	129.59	86.39	172.79	34.56	57.60	4.32
MTCC 3041	35.86	–	61.35	10.52	17.53	–

[a]–, No inhibition.

In conclusion, the indigenous bacterial probiotics possess antagonistic activity against tested antibiotic-resistant pathogens which is primarily due to production of BLIS. This property may be useful in establishment of these probiotic isolates in the competitive environment of the gut and also in improving the microbiological quality/preservation of foods. This study provides insight into antagonistic potential of indigenous probiotic isolates obtained from traditional fermented foods being consumed since ages by the people of tribal areas, i.e. Western Himalayas of the country.

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Antimalarial effect of *Tinospora cordifolia* (Willd.) Hook.f. & Thoms and *Cissampelos pareira* L. on *Plasmodium berghei*

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***Cissampelos pareira* L. and *Tinospora cordifolia* (Willd.) Hook.f. & Thoms inhibited the propagation of rodent parasite *Plasmodium berghei in vivo*. In a typical four-day experiment, the BALB/c mice were administered with ethanol extracts of *Cissampelos pareira* L. and *Tinospora cordifolia* (Willd.) Hook.f. & Thoms. The parasitaemian in untreated control group ranged between 17.31% and 30.02% whereas the root extracts of *Cissampelos pareira* L. and stem extracts of *Tinospora cordifolia* (Willd.) Hook.f. & Thoms resulted in inhibition of *Plasmodium berghei* significantly. The inhibitory properties of extracts of two plants require further studies so that the antimalarial activity is elucidated.**

Keywords: Antimalarial activity, *Cissampelos pareira*, malaria, *Plasmodium berghei*, *Tinospora cordifolia*.

MALARIA, a prominent global health hazard is caused by the protozoan parasite of the genus *Plasmodium*. Only four species of *Plasmodium*, namely *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* infect human beings. Of these *P. falciparum* and *P. vivax* cause a majority of malaria infections. About 3.3 billion people in the world are at risk of malaria. Every year, about 250 million malaria cases are reported and nearly one million deaths occur due to this disease[1]. In addition to its health toll, malaria puts a heavy economic burden on countries where it is endemic. The estimates of expenditure to tackle malaria globally in 2009 and 2010 are US$ 5.335 billion and 6.180 billion respectively which include direct costs for diagnosis, treatment and prevention[2].

Malaria elimination requires robust strategic planning for it to succeed[3]. In the absence of any effective malaria vaccine, chemotherapy plays an important role in containment of the disease but unfortunately, drug-resistant strains of *Plasmodium* have appeared against most of antimalarials introduced till date. *P. falciparum* has developed resistance to practically all the antimalarials used currently[4] and continues to increase in both intensity and geographic distribution[5]. Due to drug resistance and unavailability of malaria vaccine, there is a need of exploring the flora for antimalarial properties.

Artether, artemether and artesunate from *Artemisia annua* have effectively treated drug-resistant *P. falciparum*. Extracts from plants like *Entandro phragma*

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