International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2017; 5(5): 83-88
Received: 03-07-2017
Accepted: 04-08-2017

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Determination of anti-filarial efficacy of selected medicinal plants of combretaceae family against bovine filarial parasite *Setaria cervi*

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Abstract
The present investigation deals with the antifilarial activity study of six combretaceae family plants in terms of worm motility, MTT reduction and GST inhibition assays. The antifilarial activity was assessed by the treatment of methanolic extracts on target parasite *Setaria cervi* at doses 2.5, 5 and 10mg/ml with simultaneous use of control or untreated worms. *In vitro* worm motility and MTT reduction assay exhibited that methanolic extract of *Terminalia arjuna* was most active among all the six plant extracts showing motility inhibition at only 2hrs of incubation at 37 ºC with IC_{50} value of 1.8mg/ml. DEC standard showed IC_{50} value of 2.84mg/ml i.e. higher than the *T. arjuna* plant extract. Inhibition effect of active extracts of *T. arjuna* and *C. albidum* at 5 and 10mg/ml along with DEC standard on Glutathione-s-transferase was studied by spectrophotometric method. The methanolic extract of *T. arjuna* plant showed percentage inhibition value of 69.98% at 10mg/ml on GST of *Setaria cervi*. The GST activity was studied on both treated and untreated worms. We found that the methanolic extract of *Terminalia arjuna* contains some active constituents that selectively kill the adult parasites thereby providing maximum efficacy among all the six plant extracts and DEC standard.

Keywords: Antifilarial activity, Combretaceae, MTT (3-[4, 5dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide), Glutathione-s-transferase (GST), *Setaria cervi*

1. Introduction
Lymphatic filariasis is one of the leading causes of disfigurement and disability in endemic areas, caused by nematode parasitic species *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori* [1]. According to World Health Organization (WHO), over 1.10 billion people in 55 countries are living in areas that require preventive chemotherapy to stop the spread of infection. Globally, an estimated 25 million men suffer with genital disease and over 15 million people are afflicted with lymphoedema [2]. India is one of the endemic countries with approximately 48 million infected individuals [3], accounting for 40% of the worldwide filariasis burden [4]. Currently, Diethylcarbamazine (DEC), Albendazole and Ivermectin are the drug of choice to treat patients suffered from filariasis. These drugs are effective against circulating microfilariae but they have limited or no macrofilaricidal activity, which means repeated treatment is required over many years and the possibility that resistance may arise to the drug is a cause of concern [5, 6]. Therefore, new macrofilaricidal or faster acting drugs are needed.

In the present study, the macrofilaricidal property of the methanolic extract of six Combretaceae family plants (*Combertum albidum*, *Combretum densiflorum*, *Combretum roxburghii*, *Terminalia alata*, *Terminalia arjuna* and *Terminalia racemosa*) were evaluated against bovine filarial parasite *Setaria cervi*. Setaria is a genus of parasitic round worms that infect bovids such as pigs, camels, cattles, buffalos, horses, deer and antelope [7]. *Setaria* cervi, resembles the human bancroftian parasite in its antigenic pattern, nocturnal periodicity and similar chemotherapeutic antifilarial drug response, therefore it has been used as a model parasite for drug discovery research [8].

Combretaceae is a large family of trees, shrubs, vines and mangroves which consists of approximately 17 genera and 525 species [9]. The Combretaceae family plants occur mainly in tropical and subtropical regions internationally, with the highest diversity in Asia and Africa [10]. Two of the largest and most useful genera are Combretum, consisting of approximately 250 species of trees, shrubs and lianas, and Terminalia, consisting of approximately 150 species of trees and shrubs. Both the genus are endowed with plenty of secondary metabolites including, triterpenes, flavonoids, lignans and non-protein amino acids [11]. These secondary metabolites deal with various disease healing activities like abdominal and back pain, coughs and colds, conjunctivitis, diarrhoea and dysentery, fever, headache, heart disorders,

inflammation, leprosy, pneumoniae, sexually transmitted diseases, worms, wounds, haemorrhages, ulcers, and as a general tonic [12]. These properties of combretaceae family plants can be explored for antifilarial principles with higher efficacy and easy availability.

2. Materials and methods

2.1 Collection and Processing of plant material

Fresh leaves of six selected plant species of Combretaceae family *Combertum albidum*, *Combretum densiflorum*, *Combretum roxburghii*, *Terminalia alata*, *Terminalia arjuna* and *Terminalia racemosa* were collected from medicinal germplasm garden of Regional Plant Resource Centre, Bhubaneswar. The leaves were collected in the month of March to August. These leaves were washed in running tap water to remove dirt and then shade dried at room temperature. After complete drying, the leaves were grinded to make powder using a mechanical grinder.

2.2 Solvent Extraction

The powder leaf sample (30gm) was taken in a thimble and then extraction was started using 250ml of methanol in a soxhlet apparatus. After completion of the process, the solvent extract was concentrated using rotary evaporator (Buchi-R200) to make crude methanolic extract. The crude methanolic extract was air dried into semisolid mass and required amount was dissolved in Dimethylsulfoxide (DMSO, SRL) for further study.

2.3 Parasite collection and preparation

Adult *Setaria cervi* worms were collected from the peritoneal cavity of freshly slaughtered buffalo. The worms were washed in phosphate buffer saline (PBS, 1x, pH 7.4) to remove extraneous material and used for the assay. Then the worms were transferred immediately to RPMI-1640 medium (Hi-media, Mumbai, India) supplemented with 5% (v/v) heat-inactivated Fetal bovine serum [13].

2.4 *In vitro* motility inhibition assay

A female worm was introduced into 2ml of RPMI 1640 medium (Hi-media, Mumbai, India) taken in a 24well culture plate supplemented with 0.5% (v/v) fetal bovine serum (Himedia, Mumbai, India). The methanolic extracts of all the plant species were dissolved in DMSO to obtain 2.5, 5 and 10mg/ml concentration. The experiment was set up in triplicate along with negative control (Untreated) and vehicle (Only DMSO). After extract treatment the worms were incubated at 37 ºC in an incubator for 24hrs. The motility was observed at one hour interval up to 4 hrs and final reading was taken after 24hrs. Diethylcarbamazine (DEC) with same concentrations was taken as standard drug for this assay. The motility readings were graded as 4+ (Highly motile), 3+ (Motile), 2+ (Sluggish), 1+ (Non-motile) and 0 (Dead). After 24hrs of exposure the worms were transferred to fresh PBS (1x, pH 7.4) to check the motility of the worms whether any of the immotile worms regained motility in the post treatment period. If the worms did not revive, the condition was considered as irreversible and the concentration lethal. Each experiment was repeated three times [14].

2.5 MTT- reduction assay

Worm viability assessment study was done by *in vitro* MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] formazan reduction assay [15]. After worm motility inhibition study, the treated worms were washed in fresh PBS (1x) pH 7.4. Then the parasites were transferred to 24 well culture plates containing 0.01% MTT prepared in PBS (1x) and incubated at 37 ºC for 1hr duration. After incubation the treated worms were transferred to chilled PBS (1x) and then soaked in tissue paper. Worms were carefully transferred to 24 well culture plates containing 2ml DMSO and allowed to be at room temperature for 2hrs with occasional gentle shaking to develop blue colour. The absorbance of the resulting formazan solution was then determined at 510nm using spectrophotometer (Shimadzu, UV-1800). High value of absorption correlates with high viability of the worms. Viability of the worms was estimated as percentage inhibition in formazan formation relative to solvent controls by following formula:

Percentage of reduction = (Control – Sample) x 100/ Control

The percentage inhibition >70% was considered significant and also IC_{50} value was calculated for each extract tested on *Setaria* worm. The lower IC_{50} value denotes greater activity of the plant extracts.

2.6 Glutathione-S- transferase (GST) inhibition assay

In vitro GST activity study was conducted using GST assay kit (Himedia) with some modifications [16]. The worms were treated with methanolic extracts of the selected plants and DEC (Standard) at concentration 5 and 10mg/ml along with negative control for 24 hrs at 37 ºC. After treatment the worms were homogenized using 9ml of PBS (1x, pH 7.4) and 1 ml of 3mM EDTA on ice. The tissue homogenate then centrifuged at 10,000rpm for 10mins at 4 ºC. The supernatant was used as enzyme extract for determination of GST activity using CDNB (100mM) and GSH (100mM) using a spectrophotometer. The reaction mixture in a volume of 500µl contained 465µl assay buffer (1x), 5 µl CDNB, 10 µl GSH and 20 µl enzyme solution. The absorbance increase was measured at 340nm at 1min time interval for 5mins against blank. For calculation, the ΔA_{340nm} of blank reaction was subtracted from the ΔA_{340nm} of each sample reaction. The molar extinction of CDNB is 0.0096 μM^{-1}/ cm [17]. Protein concentration was measured by the Lowry method [18] by taking Bovine Serum Albumin (BSA) as standard. The untreated worm GST activity was compared with treated worms GST enzyme activity for the determination of the percentage of inhibition by the formula:

Percentage of inhibition = ($EA_{untreated}$- $EA_{treated}$) x 100/ $EA_{untreated}$

2.7 Statistical analysis

The results were expressed as mean ± s.d for the triplicate observations made in each observation. For comparison of means of different parameters between the test plant extracts and respective controls, Student's t-test was used. P values of <0.05 were considered as significant.

3. Results

3.1 *In vitro* antifilarial activity in terms of motility inhibition assay

Crude methanolic extracts of all the six plant species of Combretaceae family *C. albidum* (CALM), *C. densiflorum* (CDLM), *C. roxburghii* (CRLM), *T. alata* (TALM), *T. arjuna* (TARLM), *T. racemosa* (TRCLM) were assessed for antifilarial screening against bovine filarial parasite *Setaria cervi*. The methanolic extract of *T. arjuna* (TARLM) caused complete immobilization of worms at 2 to 24hrs of exposure at 37 ºC, whereas in untreated control, all the worms were

found active (Table 1). Post exposure incubation in fresh medium did not revive the worms; confirm their death due to the treatment by plant extract. All other plant extracts showed minimum activity on *S. cervi* parasites even at higher doses 10mg/ml except *C. albidum*, which showed toxic effect on worms after 3hrs of incubation at 37 ºC. DEC exhibited paralysing effect at higher dose 10mg/ml after 2hrs of post exposure in the *in vitro* condition. The results confirmed that methanolic extracts of *T. arjuna* and *C. albidum* are effective on Setaria worms than other extracts and DEC standard.

Table 1: Worm motility readings at 1hr interval up to 24hrs after treatment with methanolic extracts of six plant species of Combretaceae family with standard antifilarial drug DEC.

Name of the extract/standard	Concentration (mg/ml)	Motility readings				
		1h	2h	3h	4h	24h
CALM	2.5	4+	4+	4+	3+	2+
	5	4+	3+	1+	1+	1+
	10	4+	3+	1+	1+	1+
CDLM	2.5	4+	4+	4+	4+	3+
	5	4+	4+	4+	3+	2+
	10	4+	4+	3+	3+	1+
CRLM	2.5	4+	4+	4+	4+	4+
	5	4+	4+	4+	4+	4+
	10	4+	4+	4+	4+	3+
TALM	2.5	4+	4+	4+	4+	4+
	5	4+	4+	4+	4+	4+
	10	4+	4+	4+	4+	3+
TARLM	2.5	4+	1+	1+	1+	1+
	5	4+	1+	1+	1+	1+
	10	4+	1+	1+	1+	1+
TRCLM	2.5	4+	4+	4+	4+	4+
	5	4+	4+	2+	2+	1+
	10	4+	4+	2+	2+	1+
DEC (Standard)	2.5	4+	4+	4+	4+	1+
	5	4+	4+	4+	4+	1+
	10	4+	1+	1+	1+	1+

3.2 *In vitro* antifilarial activity in terms of MTT- reduction assay

The macrofilaricidal effect of crude methanolic extracts of Combretaceae family plants *C. albidum* (CALM), *C. densiflorum* (CDLM), *C. roxburghii* (CRLM), *T. alata* (TALM), *T. arjuna* (TARLM), *T. racemosa* (TRCLM) was further confirmed by comparing the treated worms with untreated control in terms of MTT- reduction assay. During the assay the formazan formed was extracted with DMSO and quantified colorimetrically. The best activity among extracts was found from *T. arjuna* that gave 83.80% of reduction of MTT (Table 2). All other plant extracts gave minimum reduction efficiency i.e. below 70% except methanolic extract of *C. albidum* that showed 70.04% activity. The IC_{50} values of 1.8mg/ml and 2.48mg/ml were obtained for *T. arjuna* and *C. albidum* respectively, which is much lower than IC_{50} of DEC i.e. 2.84mg/ml. Both worm motility and MTT reduction assay confirmed the macrofilaricidal activity of methanolic extracts of *T. arjuna* and *C. albidum*, which is comparable with DEC standard.

Table 2: *In vitro* macrofilaricidal activity study of methanolic extracts of six plant species of Combretaceae family in terms of MTT- reduction assay using *Setaria cervi* as test organism.

Name of the plants	Concentration (mg/ml)	% of reduction Mean ± SD	IC_{50} (mg/ml)
CALM	2.5	50.24±4.51	2.48
	5	67.16±0.56	
	10	70.04±2.4	
CDLM	2.5	33.73±1.49	4.3
	5	58.06±1.34	
	10	61.11±2.32	
CRLM	2.5	0	-
	5	0	
	10	13.48± 1.25	
TALM	2.5	0	-
	5	23.65±2.04	
	10	42.54±8.98	
TARLM	2.5	69.29±1.49	1.8
	5	81.88±1.14	
	10	83.80±3.75	
TRCLM	2.5	0	4.3
	5	58.09±1.85	
	10	62.81±5.93	
DEC (Standard)	2.5	43.96 ± 3.05	2.84
	5	62.84 ± 2.05	
	10	79.22 ± 3.1	

Data analysed in triplicate. $P<0.05$ considered as significant.

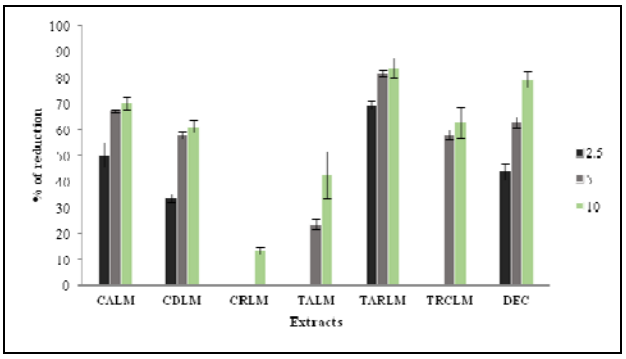

Fig 1: Adult worm mortality study in-terms of MTT- reduction assay at different doses using methanolic extracts of six plants *C. albidum* (CALM), *C. densiflorum* (CDLM), *C. roxburghii* (CRLM), *T. alata* (TALM), *T. arjuna* (TARLM), *T. racemosa* (TRCLM). DEC was used as standard in this experiment.

3.3 GST inhibition assay

Glutathione-s-transferase enzyme activity was assessed spectrophotometrically by using CDNB and GSH substrates. The active methanolic extracts of *T. arjuna* (TARLM) and *C. albidum* (CALM) at 5 and 10mg/ml concentrations were used for GST inhibition assay. The potent GST inhibition activity was obtained by the application of *T. arjuna* methanolic extract with percentage inhibition value of 69.98% at 10mg/ml. The GST activity was inhibited from untreated control worms to treated in a significant manner. The GST activity of 70.275 ± 6.67 µm/ml/min was obtained for untreated control worms, whereas in *T. arjuna* methanolic extract treated worms the GST activity was found to be 21.09 ± 5.89 and 33.07 ± 7.36 µm/ml/min at 10 and 5mg/ml doses respectively (Table 3). DEC also showed inhibitory activity on GST enzyme at the same doses and this can be a comparable with each other.

Table 3: Effects of methanolic extracts of most active plant *C. albidum and T. arjuna* on GST enzyme activity of *Setaria cervi* adult worms after 24hrs of treatment

Name of the extract/ standard	Concentration (mg/ml)	GST specific activity (µM/ml/min)	% of Inhibition	Protein content(mg/ml)
CALM	5	57.29 ± 0.81	18.47%	1.75 ± 0.21
	10	54.03 ± 0.37	23.11%	1.34 ± 0.35
TARLM	5	33.07 ± 7.36	52.94%	1.44 ± 0.31
	10	21.09 ± 5.89	69.98%	1.76 ± 0.81
DEC (Standard)	5	40.03 ± 4.14	43.03%	1.51 ± 0.6
	10	21.48 ± 6.44	69.43%	1.42 ± 0.19

Data analysed in triplicate. P<0.05 considered as significant.

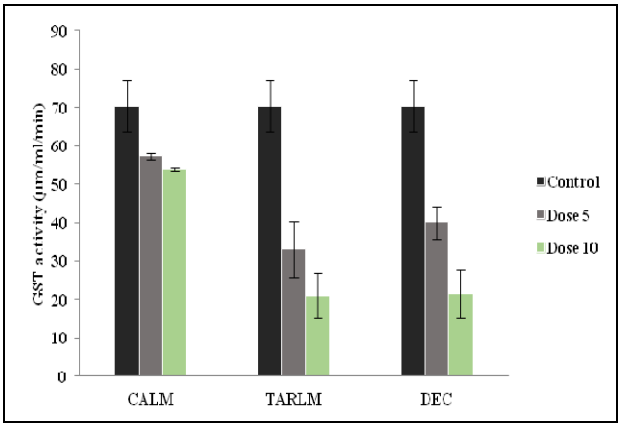

Fig 2: Antifilarial study in terms of GST enzyme inhibition using active methanolic extract of *C. albidum, T. arjuna* with DEC (Standard) on GST activity of Setaria adult worms.

4. Discussion

In the present study, the methanolic extract of *Terminalia arjuna* found most active among six plants of combretaceae. It showed promising macrofilaricidal activity on Setaria parasites in terms of worm motility and MTT reduction assay. The methanolic extracts of *T. arjuna* exerted greater inhibitory effect on Setaria worms comparatively at faster rates than other plant extracts used in this assay. It paralysed the worms significantly at only two hours of incubation using methanolic extracts at doses 2.5, 5 and 10mg/ml. MTT-reduction assay as a parameter of assessing cell viability also gave conclusive result over worm motility assay where worms may not be irreversibly damaged but merely paralysed and not able to recover. MTT assay results confirmed that worms were dead by the action of methanolic extracts of *T. arjuna* at doses 2.5, 5 and 10mg/ml and it was well compared with untreated worms. The IC_{50} value of methanolic extract of *T. arjuna* was found to be 1.8mg/ml, which is lower than all the extracts and also DEC standard used in this assay. DEC showed IC_{50} value of 2.84mg/ml.

Similar experiments have been assessed by using various medicinal plant extracts on Setaria parasites for evaluating macrofilaricidal activity. The alcoholic extracts of the leaves and seeds of *Psoralea corylifolia* at concentrations of 160, 30, and 150, 20 mg/ ml, caused the inhibition of spontaneous movements of the whole worm and the nerve muscle preparation of *S. cervi*, resulting in paralysis of the worms with LC50 and LC90 value of 15 and 25 mg/ml, 12 and 18 mg/ml respectively for the alcoholic extracts of leaves and seeds [19]. In similar studies, the crude methanolic extract of *Hibiscus mutabilis* (confederate rose) and the isolated bioactive molecule "ferulic acid" were tested against bovine *S. cervi* [20]. The authors reported that both the extract and the bioactive molecule "ferulic acid" showed significant microfilaricidal as well as macrofilaricidal activities against *S. cervi*. Mathew *et al*., 2008 studied the effect of methanolic extract of *Trachyspermum ammi* fruits against adult bovine filarial *Setaria digitata* worms at a concentration of 0.01–0.5 mg/mL for a period of 24–48 hrs and found that both the crude extract and the active fraction showed significant activity against the adult *S. digitata* by both worm motility and reduction assays [21]. Patra *et al*., 2009 reported the antifilarial activities of methanolic and aqueous extracts of *Excoecaria agallocha* leaf against *S. digitata* were dose dependent at concentrations of 10, 50, and 100 *µ*g/mL for 24 hrs at 37 ºC in 5% CO_2 incubation. The study showed reduction in percentage of motility by about 20, 60, and 83% respectively [22]. It was found that the antifilarial property of natural extracts and compounds has been explored in various medicinal plants but there were no such information obtained about the antifilarial potential of Combretaceae family plants. So this is probably a new report of the macrofilaricidal property of medicinal plants belong to Combretaceae family.

GST enzyme activity study in treated and untreated worms also gave a clear idea about drug activity. The inhibition of

GST enzyme of *Setaria cervi* by the plant extracts implied that the crude extracts have potential antifilarial activity on parasites. The crude cytosolic enzyme obtained from methanolic extract of *T. arjuna* treated *Setaria cervi* exhibited GST activity of 21.09 ± 5.89 µM/ml/min and 33.07 ± 7.36 µM/ml/min at 10 and 5mg/ml respectively (Table 3). The untreated worms showed GST activity of 70.275 ± 6.67 µM/ml/min. This clearly indicated that GST activity has been inhibited by the methanolic extract of *T. arjuna* after 24hrs of treatment. This inhibitory potential of methanolic extract of *T. arjuna* could be comparable with DEC activity on GST enzyme. Previous reports on antifilarial potential of plant extract and their isolated compounds also provided strong clues regarding suppression of GST enzyme *in vitro*. Curcumin is one of the most studied chemopreventive agent extracted from *Curcuma longa* L. is a strong GST inhibitor in human melanoma cells in which GST P1-1 is the major isoform [23]. Similarly, plumbagin is also a natural compound extracted from *Plumbago rosea* exhibiting filarial GST inhibition and antifilarial activity [24]. This is also supported by the reports of Coruh *et al*., (2007a, b) whose work on *Gundelia tournefortii*, *Prangos ferulacea* (L.) Lindl., *Chaerophyllum macropodum* Boiss. and *Heracleum persicum* Desf. extracts showed great inhibition on glutathione-S-transferase activity [25, 26]. Quinine, isolated from Cinchona bark is also a well-known example of covalent inhibitor of GST enzymes [27].

Glutathione S-transferase plays an important role in detoxification of xenobiotics in filarial parasites [28-32]. Its inhibition from the parasites by various plant extracts and isolated compounds pave the way towards development of potential chemotherapeutic strategy to combat vector borne disease like filariasis.

5. Conclusion

From our result it confirmed that the methanolic extract of *T. arjuna* plant contain bioactive agents that have effective macrofilaricidal potential. This suggests that further work on this plant for isolation of active principles can be a great strategy to overcome tremendous concern in combating filariasis.

6. Acknowledgement

Authors are thankful to Forest and Environment Department of Odisha for providing financial support during the entire research process.

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