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Research Article

Macrofilaricidal Activity of Leaf Extracts of *Rauvolfia tetraphylla* L. Against Bovine Filarial Parasite *Setaria cervi*.

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ABSTRACT

Leaf extracts of *Rauvolfia tetraphylla* were evaluated for antifilarial potential using *Setaria cervi* as target parasite. Four extracts were tested these were hexane, chloroform, acetone and methanol. The activity was assessed by means of worm motility assay, MTT reduction and GST enzyme inhibition assay using female worms of *Setaria cervi*. DEC was used as a standard drug. Methanol extract exhibited significant activity among all the extracts with percentage of reduction 89.28% at 10mg/ml concentration. DEC was comparitively less active than methanolic extract with 79.22% activity at the same dose. The IC_{50} value for methanolic extract was found to be 0.03mg/ml, which was better than the standard DEC i.e. 2.84mg/ml. The methanol extract significantly inhibited the GST enzyme activity of Setaria worms as compared to untreated worms with percentage of inhibition value of 56.41% at 10mg/ml. The most active methanol extract was finally explored for cell viability study by using trypan blue dye exclusion test. The methanolic extract significantly killed the oocytes of Setaria worms as all the oocytes stained blue in treated worms and unstained in control oocytes. The findings indicate that the methanolic extract has potent macrofilaricidal ativity.

Keywords: *Rauvolfia tetraphylla*, macrofilaricidal activity, *Setaria cervi*, Worm motility, MTT-reduction, GST inhibition, Trypan blue.

INTRODUCTION

Rauvolfia tetraphylla is a shrub belonging to Apocynaceae family which is well known for tremendous medicinal potential[1]. The roots of this plant are frequently used in India as a substitute for *Rauvolfia sepentina* for medicinal purposes[2]. The latex of this plant is said to be cathartic, diuretic, emetic and expectorant[3]. The plant is commonly used in the treatment of malaria and also as reputed remedies for snake bites in Guatemala[4]. The roots of this plant yield alkaloids like reserpine, used in making various allopathic medicines and deserpidine, which is an antihypertensive and tranquilizer[5]. As plant has shown its potential in parasitic disease like malaria, it was considered worthwhile to evaluate its potential for another tropical parasitic disease filaria.

In this study, effect of leaf extracts of *Rauvolfia tetraphylla* was explored against bovine filarial parasite *Setaria cervi* which resembles the human bancroftian parasite in its nocturnal periodicity, antigenic pattern and same chemotherapeutic antifilarial drug response towards filarial drugs[6]. Thus, activity against the parasite might provide lead for developing new antifilarial drug for lymphatic filariasis as well.

MATERIALS AND METHODS

Collection and Processing of Plant Material

The fresh leaves of *Rauvolfia tetraphylla* were collected from medicinal germplasm garden of Regional Plant Resource Centre (RPRC), Bhubaneswar. Leaves were washed thoroughly under running tap water to remove dust followed by drying in shade at room temperature. After complete drying, these leaves were made into coarse powder using a mechanical grinder. The powder leaf sample (50g) was extracted with hexane, chloroform, acetone and methanol solvents successively by soxhlet extraction technique. The liquid extracts were concentrated using rotary evaporator (Buchi R-200) to get concentrated extract. Semi solid extracts were stored in tight screw capped vials till further use.

Collection of Experimental Organism

Samples were procured from Nandankanan Slaughter House, Government of Odisha, Bhubaneswar. Adult *S. cervi* worms were collected from peritoneal cavity of freshly slaughtered buffalo and washed in PBS (1x, pH 7.4) to remove blood and tissue debris. Then the worms were transferred immediately to RPMI-1640 medium and supplemented with 5% (v/v) heat-inactivated Fetal bovine serum.

Assessment of Antifilarial Activity

Worm motility inhibition assay

The *in vitro* worm motility inhibition assay was performed by standard protocol[7]. Screening was done at concentration ranging from 2.5 to 10mg/ml for all the extracts and Standard drug. One adult female worm was introduced into each vial and incubated at 37ºC for 24hrs. Three replicates each were set up for both test and controls. After one hour difference motility readings were taken up to 4 hours as per the gradation as follows: 4+ -

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Table 1: Worm motility readings at 1hr interval up to 24hrs after treatment with extracts/standard.

Extracts	Concentration (mg/ml)	Motility Readings in different time intervals				
		1h	2h	3h	4h	24h
Hexane	2.5	4+	4+	4+	4+	4+
	5	4+	4+	4+	4+	4+
	10	4+	4+	4+	4+	2+
Chloroform	2.5	4+	4+	3+	3+	2+
	5	4+	4+	3+	3+	1+
	10	4+	4+	1+	1+	1+
Acetone	2.5	4+	4+	4+	3+	2+
	5	4+	4+	4+	2+	1+
	10	4+	4+	4+	1+	1+
Methanol	2.5	4+	1+	1+	1+	nil
	5	4+	1+	1+	1+	nil
	10	4+	1+	1+	1+	nil
DEC (Standard)	2.5	4+	4+	4+	4+	1+
	5	4+	4+	4+	4+	1+
	10	4+	1+	1+	1+	1+

All the experiments were repeated thrice, n=3

Table 2: *In vitro* macrofilaricidal activity study of different extracts of *R. tetraphylla* in terms of MTT-reduction assay using *Setaria cervi* as test organism.

Extracts	Concentration (mg/ml)	% of reduction (Mean ± S.D)	IC_{50} (mg/ml)
Hexane	2.5	0	
	5	0	-
	10	14.45 ± 1.8	
Chloroform	2.5	42.97 ±2.32	
	5	60.04 ±1.51	2.91
	10	71.47 ±1.47	
Acetone	2.5	38.16 ±0.69	
	5	55.09 ±0.98	4.53
	10	69.74 ±0.61	
Methanol	2.5	75.92±3.8	
	5	80.63±3.8	0.03
	10	89.28±2.2	
DEC	2.5	43.96± 3.05	
	5	62.84± 2.05	2.84
	10	79.22 ± 3.1	

All the experiments were repeated thrice, n=3, P<0.05 considered as significant.

highly motile, 3+ - motile, 2+ - sluggish, 1+ - non motile, nil – dead. The final reading was taken after 24hrs. After exposure, the worms were transferred to fresh PBS (1x) without the test solution to find out whether any of the immotile worms regained motility after treatment period in drug free medium. If the worms did not revive, the condition was considered as irreversible and the concentration lethal. Each experiment was repeated three times.

MTT-formazan reduction assay

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 37ºC for 1hr duration. After incubation the treated worms were transferred to chilled PBS (1x) and 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 extract the colour developed. The absorbance of the resulting formazan solution was then determined at 510nm using spectrophotometer. 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:

$$\text{Percentage inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

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.

Glutathione-S- transferase (GST) inhibition assay

In vitro GST activity study was conducted using GST assay kit (Himedia) with some modifications[8]. The worms were treated with extracts and DEC (Standard) at concentration 5 and 10mg/ml along with negative control for 24 hrs at 37ºC. 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 4ºC. 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 $\mu M^{-1}/cm$[9].

Trypan blue dye exclusion test

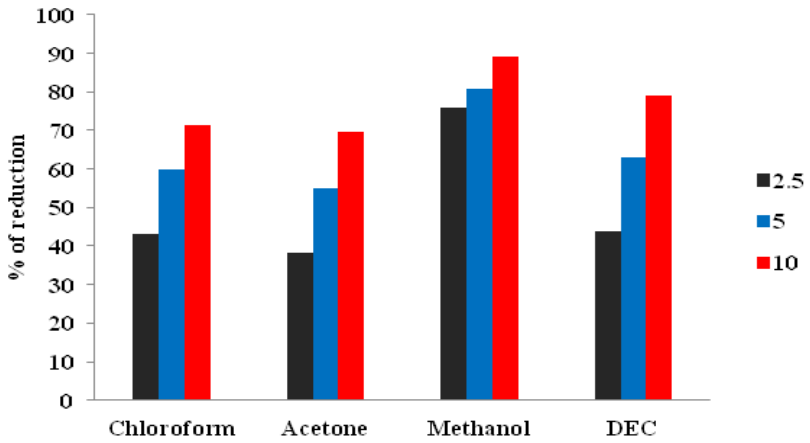

Figure 1: Adult worm mortality study in-terms of MTT- reduction assay at different doses using extracts/ standard.

Table 3: Effects of extracts of *R. tetraphylla* on GST enzyme activity and GSH content of *Setaria cervi* adult worms after 24hrs of treatment.

Extracts	Concentration (mg/ml)	GST specific activity (µm/ml/min)	% inhibition	Protein Content (mg/ml)
Chloroform	5	68.24 ± 1.11	2.89%	1.92 ± 0.11
	10	51.29 ± 0.87	27.01%	1.9 ± 0.23
Acetone	5	52.19 ± 0.9	25.73%	1.44 ± 0.8
	10	48.12 ± 0.65	31.52%	1.76 ± 0.35
Methanol	5	43.61 ± 1.8	37.94%	1.71 ± 0.91
	10	33.2 ± 1.4	52.75%	2.07 ± 0.63
DEC	5	40.03 ± 4.14	43.03%	1.51 ± 0.6
	10	21.48 ± 6.44	69.43%	1.42 ± 0.19

All the experiments were repeated thrice, n=3. P<0.05 considered as significant.

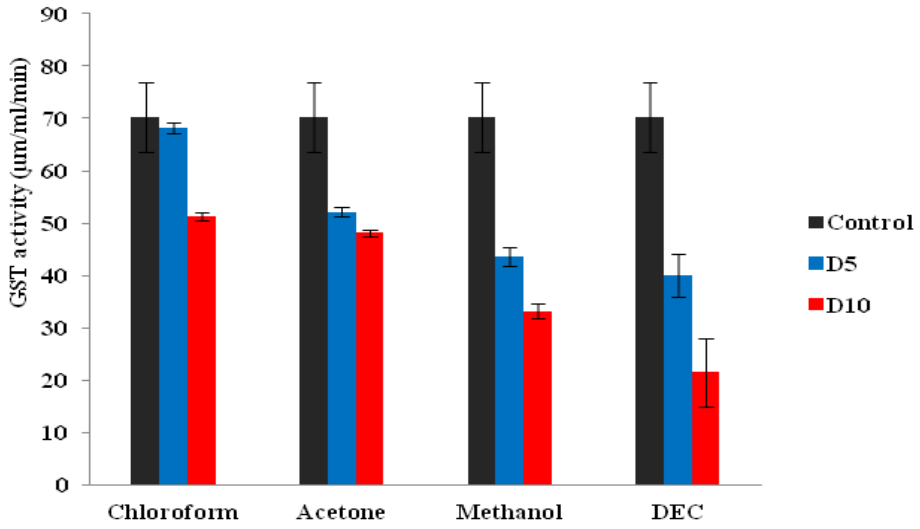

Figure 2: Effects of extracts of *R. tetraphylla* on GST activity of Setaria adult worms using control and treated groups.

Cell viability study was determined by trypan blue dye exclusion test on worm oocytes. Viability of oocytes was analyzed by 1% Trypan blue dye (Spectrochem Pvt. Ltd, Mumbai) dissolved in PBS (1x and pH 7.4). After 24 h of incubation, the control and treated (2.5 to 10 mg/ml of extract) worms were taken on glass slides and punctured with needle to remove oocyte from its body. Then 100µl of 1% trypan blue dye was injected on it and covered with cover slip. After that the slides were analyzed under phase contrast microscope (Olympus, BX51)[10].

RESULTS AND DISCUSSIONS

Four leaf extracts of *Rauvolfia tetraphylla* namely, hexane, chloroform, acetone and methanol were explored for their macrofilaricidal activity in terms of worm motility inhibition, MTT- reduction, GST enzyme

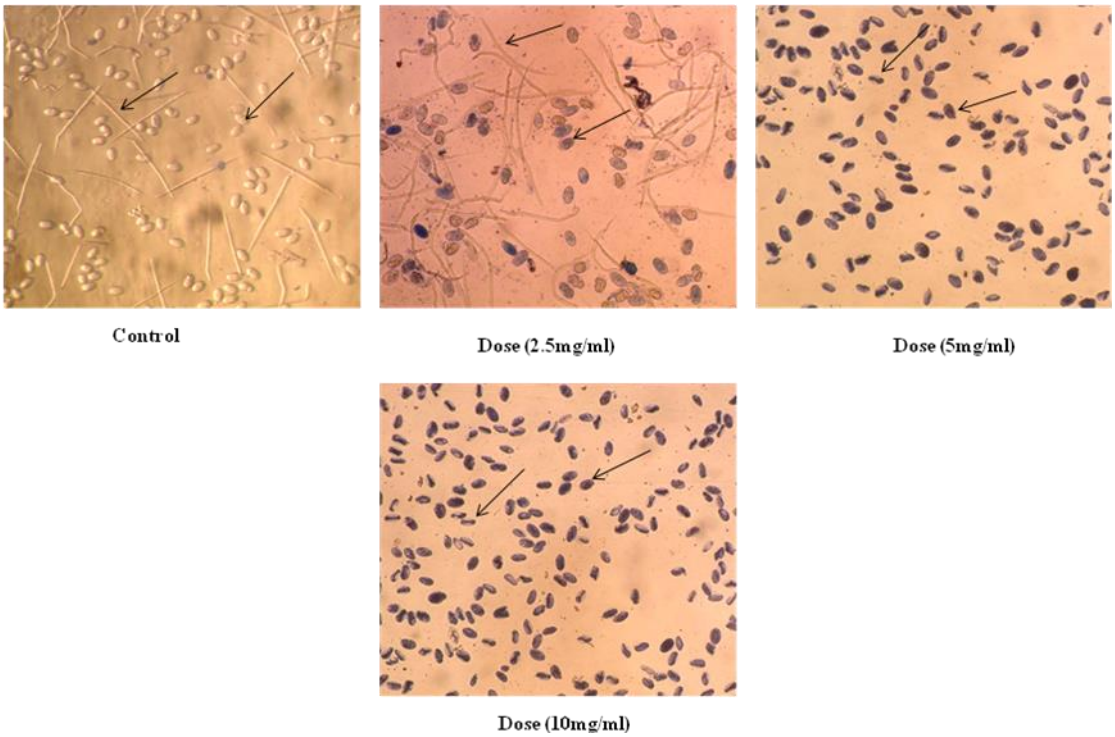

Figure 3: Apoptotic features of the oocytes of *Setaria cervi* worms after 24hrs of active methanolic extract treatment at different doses examined under microscope at 40x magnification, (Fig. a) Control showing unstained oocytes, (Fig. b, c & d) treatment doses at 2.5mg/ml, 5mg/ml & 10mg/ml showing blue coloured oocytes.

inhibition and dye exclusion test using *Setaria cervi* as target parasites for antifilarial activity. It is practically impossible to procure *Wuchereria bancrofti* a human parasite, So, a nematode parasite resembling Wuchereria in its antigenic pattern, similar nocturnal periodicity and same chemotherapeutic response towards antifilarial drugs has been used for antifilarial drug development research namely *Setaria cervi*[6].

As can be observed from Table 1, methanol extract of *R. tetraphylla* significantly reduced worm's motility within 2nd hour of incubation at concentrations 2.5, 5 and 10mg/ml whereas DEC, complete immobilization of worms was after 4hrs of incubation higher dose 10mg/ml. This supported the fact that standard drug has insignificant activity against adult worm as also reported before[11]. Remaining extracts such as chloroform and acetone showed moderate activity on Setaria worms as depicted in Table 1. Control worms were active and alive till the end of the test period i.e. 24hrs. Post exposure incubation in fresh medium (without extract solution) for half an hour did not revive the worms in active extract (methanol); confirming their death due to extract treatment.

MTT reduction assay for macrofilaricidal activity

The macrofilaricidal effect of extracts of *R. tetraphylla* and DEC (standard) on *Setaria cervi* was confirmed by comparison of treated worms with untreated controls in terms of MTT-formazan colorimetric assay. The light yellow coloured MTT solution changed to dark blue colour complex after exposure to live worms by mitochondrial reductase enzyme. The formazan formed is extracted with DMSO and quantified colorimetrically[12]. The methanol extract of *R. tetraphylla* showed significant MTT reduction activity at doses 2.5, 5 and 10mg/ml as compared to DEC (standard) and other extracts. The highest percentage of reduction i.e. 89.28% obtained at 10mg/ml. While for DEC the MTT reduction value was found to be 79.22% at 10mg/ml (Table 2,Fig 1). The inhibitory concentration at 50% (IC_{50}) was also calculated for both sample and standard using MS excel by plotting concentration versus percentage of reduction. The IC_{50} value for methanol extract was found to be 0.03mg/ml which is much lower than IC_{50} value for DEC i.e. 2.84mg/ml. The hexane extract showed no activity on Setaria worms, where as chloroform and acetone extracts exhibited good effect on Setaria worms with 71.47% and 69.74% respectively. The *in vitro* worm motility study and MTT-formazan reduction assay clearly indicated that methanol extract of *R. tetraphylla* is most effective against *Setaria cervi* parasite as it gave maximum motility inhibition after 1hr incubation period even at lower doses and significant MTT reduction values i.e. 89.28% as compared to DEC standard. DEC is a standard drug used against filariasis to kill microfilariae circulating in the blood. But unfortunately, this drug is mainly microfilaricidal rather than macrofilaricidal, which means that repeated treatment is required over many years, and the possibility that resistance to them may develop is a cause for concern and also exhibits various side effects such as nausea, vomiting and headache[13].

GST inhibition assay

Glutathione s-transferase activity of the worm cytosolic fraction was evaluated after treating the worms with active extracts of *R. tetraphylla* and DEC (standard). The

result showed that GST enzyme activity reduced in treated worms as compared to control or untreated worms i.e. 33.2±1.4, 43.61±1.8µm/ml/min for 10 and 5mg/ml concentration with the percentage of inhibition 37.94% and 52.75% respectively in methanol extract as compared to GST activity of untreated worms i.e. 70.275±6.67 µm/ml/min (Table 3). In DEC treated worms the GST enzyme activity was found to be 21.48±6.44, 40.03±4.14µm/ml/min for 10 and 5mg/ml concentration with percentage of inhibition values 43.03% and 69.43% respectively (Figure 3). The GST enzyme inhibition assay also provides a clear idea about the inhibitory activity of the extracts on the target enzyme. GST is a phase II detoxification enzyme that is the crucial member of the antioxidant family that controls or is directly involved in nematode survivability, has been reported in *S. cervi*[14]. It provides protection to external assaults and endogenous cyto- and genotoxins by catalyzing nucleophilic addition of the tripeptide GSH to them. In this present study methanol extract exerted profound inhibitory effect on GST activity of *Setaria cervi* as compared to control or untreated worms.

Cell death analysis in terms of dye exclusion test

Trypan blue dye exclusion test was performed for most active methanol extract as it exhibited maximum reduction values in terms of MTT, GST and GSH level significantly. It was confirmed that oocytes were dead after treatment with active methanol extract of *R. tetraphylla*, as they stained blue. All the viable oocytes were visualized in control worms as they excluded the dye and remained unstained. The treated oocytes were stained blue in dose dependent manner showing death of organism. The treatment of methanol extract at 2.5, 5 and 10mg/ml exhibited completely blue stained oocytes and were characterized by shrinkage, distorted membrane and deformities. The apoptosis feature of oocytes was confirmed by trypan blue dye exclusion method. Trypan blue is a vital stain that leaves nonviable cells with a distinctive blue colour, while viable cells appear unstained because viable cells have intact cell membranes hence do not take in dye from the surrounding medium[15].

Overall it can be concluded that methanol extract exhibited better activity in comparison to the standard drug DEC in terms of all the assays conducted in the study. This study has provided lead in the form of one leaf extract for future experimental work in the field of isolation of active potent molecule for antifilarial activity.

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REFERENCE

1. Wong SK, Lim YY, Abdullah NR, Nordin FJ. Assessment of antiproliferative and antiplasmodial activities of five selected Apocynaceae species. *BMC Complementary and Alternative Medicine*. 2011;11:3. doi:10.1186/1472-6882-11-3.
2. Ganga Rao B, Umamaheswara Rao P, Sambasiva Rao E, Mallikarjuna Rao T, Praneeth.D VS. Evaluation of *in-vitro* antibacterial activity and anti-inflammatory activity for different extracts of *Rauvolfia tetraphylla* L. root bark. *Asian Pacific Journal of Tropical Biomedicine*. 2012; 2(10):818821. doi:10.1016/S2221-1691(12)60235-4.
3. Tekwu EM, Bosompem KM, Anyan WK, et al. In vitro assessment of anthelmintic activities of *Rauwolfia vomitoria* (Apocynaceae) Stem Bark and Roots against parasitic stages of *Schistosoma mansoni* and Cytotoxic Study. *Journal of Parasitology Research*. 2017;2017:2583969. doi:10.1155/2017/2583969.
4. Willcox ML, Bodeker G: Traditional herbal medicines for malaria. BMJ 2004; 329: 1156-1159.
5. Pepin J-M, Therien B, Vitye B, Lemieux G, Genest J. Treatment of Arterial Hypertension with Recanescine : A New Alkaloid Isolated From Rauwolfia . *Canadian Medical Association Journal*. 1957;76(6):486-488.
6. Al-Abd NM, Nor ZM, Mansor M, Hasan M, Kassim M. Antifilarial and Antibiotic Activities of Methanolic Extracts of *Melaleuca cajuputi* Flowers. *The Korean Journal of Parasitology*. 2016;54(3):273-280. doi:10.3347/kjp.2016.54.3.273.
7. Mathew N, Paily PK, Abidha VP, Kalyanasundaram M, Balaraman K. Macrofilaricidal activity of the plant Plumbago indica/rosea *in vitro*. Drug Dev. Res. 2002; 56: 33-39.
8. Ahmed R, Srivastava AK. Cytosolic and microsomal glutathione-s-transferase from bovine filarial worms Setaria cervi. J Parasitol 2007; 93:1285-90.
9. Landmann F, Voronin D, Sullivan W, Taylor MJ. Anti-filarial Activity of Antibiotic Therapy Is Due to Extensive Apoptosis after *Wolbachia* Depletion from Filarial Nematodes. Schneider DS, ed. PLoS Pathogens. 2011;7(11):e1002351. doi:10.1371/journal.ppat.1002351.
10. Sanfilippo S, Canis M, Ouchchane L, et al. Viability assessment of fresh and frozen/thawed isolated human follicles: reliability of two methods (Trypan blue and Calcein AM/ethidium homodimer-1). Journal of Assisted Reproduction and Genetics. 2011; 28(12):1151-1156. doi:10.1007/s10815-011-9649-y.
11. Harinath BC, Reddy MVR, Bhunia B, et al. Filaria associated clinical manifestations in children in an endemic area and morbidity control by immunomonitoring and optimal DEC therapy: Sevagram experience. Indian Journal of Clinical Biochemistry. 2000; 15(Suppl 1):118-126. doi:10.1007/BF02867551.
12. Comley JCW, Rees MJ, Turner CH, Jenkins DC. Colorimetric quantitation of filarial viability. Int. J. Parasitol 1989; 19 (1): 77–83.
13. Amarillo MLE, Belizario VY, Sadiang-abay JT, Sison SAM, Dayag AMS. Factors associated with the acceptance of mass drug administration for the elimination of lymphatic filariasis in Agusan del Sur,

Philippines. Parasites & Vectors. 2008;1:14. doi:10.1186/1756-3305-1-14.

14. Saeed M, Baig MH, Bajpai P, Srivastava AK, Ahmad K, Mustafa H. Predicted binding of certain antifilarial compounds with glutathione-S-transferase of human Filariids. Bioinformation. 2013;9(5):233-237. doi:10.6026/97320630009233.
15. Prier RC, Lammie PJ. Differential regulation of in vitro humoral and cellular immune responsiveness in Brugia pahangi-infected jirds. Infection and Immunity. 1988;56(12):3052-3057.