BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, June 2021. Vol. **18**(2), p. 395-401

Antifilarial Screening and Oxidative Role of Isolated Fraction from *Aegle Marmelos* Corr. Leaves Extract

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http://dx.doi.org/10.13005/bbra/2926

(Received: 03 June 2021; accepted: 17 August 2021)

In the present work antifilarial active fraction was isolated from the leaves Chloroform extract of Aegle marmelos Corr. evaluated in vitro for antifilarial activity and studied the possible oxidative role against Setaria cervi parasite. Antifilarial study was carried out with isolated fractions by worm motility and MTT assays. Complete parasite motility inhibition was observed at 0.002 to 0.08 mg/mL in motility assay and in MTT assay plant fraction gave > 50% reduction 58.9, 74.6 and 97.2% at concentrations 0.02, 0.04 and 0.08 mg/mL at 10, 6 and 2 hours incubation period respectively ($p < 0.05$). Inhibitory concentration (IC50) was found to be 0.015 mg/mL. Oxidative parameters levels for MDA, Carbonyl content and Nitric oxide were identified as antifilarial activity achieved. The level of oxidative parameters was calculated in dose dependent manners as compared to the control level. The antifilarial activity of isolated fraction is associated with the oxidative mechanism in this study.

Keywords: Antifilarial; Aegle marmelos; leaves; Oxidation.

Filariasis appears in the tropical and subtropical areas of the world. Filariasis is mainly infected by filarial nematodes *Wuchereria bancrofti and Brugia malayi parasite*. It is spread by mosquito vectors[1]. It is an important health problem, which affects more than 100 million populations throughout the world. In lymphatic filariasis major side effects are swelling in lower legs and disfigure in prevalent sites, which lead to considerable social, economical and psychological effects. In India, 48 million populations are infected from filariasis and approximately 45% of its 1 - billion persons are lives in filariasis known areas[2] and calculated for 40% of global disease burden[3]. Yearly loss cause to a billion dollars by this disease, as per the various social and economic studies[4].

World health organization has documented as a main community health crisis in filariasis endemic areas. It is specifically documented in its TDR mandate and initiated a world agenda for filaria disease eradication (GPELF)[5]. Antifilarial known drugs Albendazole, DEC and Ivermectin are recently giving to a population for this disease[1]. The drugs can not to kill adult worms. Adult worms survive many years in infected peoples[6]. So, it needs to formulate a potent and safe drug to treat and remove the filarial parasite. Herbal resources contain a variety of plant active molecules which

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is utilized the most in herbal therapeutics. WHO has recommended traditional medicine as a key substitute resource for potent filarial disease in his TDR plan[7], but the lack of scientific study is the most important lacuna to use conventional therapeutics.

Aegle marmelos Corr. belongs to *Rutaceae* family. A middle sized slender aromatic armed plant. It is spread all over the India, from sub - Himalayan forest, Bengal, Central and SoutInern part of India and Burma[8.] This plant has anti-inflammatory, antipyretic and analgesic properties[9], antithyroid, antioxidative and antihyperglycemic activity[10], hypoglycemic and anti-hyperglycemic activity[11], antihyperglycemic and antidyslipidemic[12], analgesic activity[13], acute and sub acute toxicity studies[14], anti fertility[15], hepatoprotective effect[16], Insecticidal activity[17], Immunomodulatory activity[18] and protective effect[19].

Significant antifilarial activity[20] and oxidative status identified against microfilaia[21]. Current data has proved about polyphenols are the main molecules found in various flavonoids and alkaloids compounds, which might be worked as pro-oxidants[22]. In apoptosis, oxidative event is crucial for parasite death[23].

Looking at these viewpoints, in the current investigation, evaluated antifilarial study of extracted fraction from *Aegle marmelos* Corr. leaves and screened the probable mechanism of the herbal compound to identify the probable relation of oxidative stress rationale in this study.

MATERIALS AND METHODS

Procurement of plant materials

Plant leaves of *Aegle marmelos* Corr. were collected from the natural field of local areas of Bhopal. It is taxonomically identified by Botany Department, Safia Science College, Bhopal. The voucher specimen no. 418/Bot./Safia/2012 was given by the department.

Extraction

Aegle marmelos Corr. leaves were extracted in petroleum ether (60ºC - 80ºC), $CHCl_3$ and Methanol respectively[23, 24.]

Fractionation

To know the number of phytochemical compounds present in chloroform extract, thin layer chromatography was performed by using pure Ethyl acetate as a mobile phase and detected with Anisaldehyde and Sulphuric acid spraying solution. Further, the extract was passed through column chromatography. It was performed by using pure Ethyl acetate as a mobile phase and the fraction was isolated[23, 24, 26].

Parasite

Parasite *Setaria cervi* were collected from slaughtered buffalo. The Parasite was washed in 0.85% saline[27.]

***In-vitro* motility inhibition assay**

Parasites were transferred to DMEM media with 0.01% Strepto-penicillin and 10% heat-fetal calf serum. *Aegle marmelos* leaves fraction concentration 0.002 - 0.08 mg/mL was used for testing. In each petri plate two parasites (Female & Male) were taken. Plates were incubated in a CO_2 incubator (5%) at 37ºC for 24 hrs and after 2 to 24 hrs interval motility was observed. Each concentration was tested thrice[28, 20.]

MTT assay

MTT assay was used to check the activity of plant fraction against parasites by the methodology given by Strote G., 1998. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is yellow coloured dye, reduced by certain cellular enzymes to the blue colour formazan product. Only female parasites have been taken for this assay. The worms were incubated in 0.5 mL PBS containing 0.25 mg/mL MTT upto 30 minutes. Further worms were incubated in DMSO for 1 hour in shaking conditions to extract formazan. OD was taken at 492 nm in ELISA reader. DMSO exposed female parasites was established as a positive control. At 56ºC heat killed negative control was taken and treated with MTT dye. Worms viability was estimated[29] by the formula-

$$\% \text{ inhibition parameters} = 100 - [(T - H) / (C - H)] \times 100$$

Thus T, C, and H are O.D. values of formazan developed with test, control and heat killed parasites.

MDA estimation

To MDA standards (2.5 - 40 nM/mL), 0.5 ml of parasite culture supernatants of extract fraction and 20% TCA (2.5 mL) + 0.67% TBA (1 mL) were taken and vortex mixed. Mixtures

heated in a hot water bath till 30 min. After cooling, chromogen was extracted in n - butanol and centrifuged machine at 3,000 rpm for 10 minutes for organic phase was separation. Absorbance was taken at 530 nm. Concentrations of MDA culture supernatant were calculated (nmol MDA/mL)[30].

Protein Carbonylation Assay

For protein carbonylation estimation[31] Culture supernatants were reacting with TCA (10%) to react with 10 mM (0.5 mL) of DNPH in 2M HCl for 1 hr at room temperature. Centrifuge the precipitated ice-cold 10% TCA, at 5000 rpm till 5 minutes and washed three times with ethylacetate - ethanol mixture. Washed pellets are dissolved in protein dissolving solution (1.5 ml) and incubated at 37^0C for 10 minutes. O.D. were taken at 370 nm against HCl (2M).

Nitric Oxide Assay

Nitric oxide levels were estimated in parasite culture supernatants[32]. Griess reagent (100ìL) was added in culture supernatant (100 ìL) wells of ELISA plats and incubated for10 min. Optical density was taken at 542 nm. Standard graph (0.005 to 0.08 µM/mL) was plotted and Nitric oxide levels in culture were calculated.

Statistical analysis

Analysis was carried out to compare the results of test and controls. For this student's t test was used. $P < 0.05$ was measured as a significant value.

RESULT

Fraction isolation

Extract fraction was dried at reduced pressure.

***In vitro* motility inhibition assay**

Extract fraction was tested against *Setaria cervi* for antifilarial activity. Concentrations 0.002 to 0.08 mg/mL inhibits the motility of parasite at

Table 1. Antifilarial activity of fraction against filarial worm *in vitro* motility inhibition

Test concentration of compound (mg/mL)	Incubation time (end point) in hrs	Worm motility inhibition (Test)	Worm motility inhibition (Control)
0.002	24	#	†
0.005	20	#	†
0.01	14	#	†
0.02	10	#	†
0.04	6	#	†
0.08	2	#	†

[#]Completely Immotile worm [†]Completely motile worms.

Table 2. Antifilarial activity of fraction against filarial worms in term of MTT assay

Sample	Incubation time (In hrs.)	Test concentrations (mg/mL)	Absorbance at 492 nm (mean ± SEM)	% reduction to solvent control[C], heat killed[H] & treated parasite[T]	IC50 (mg/mL)
[C]Control	24	-	0.996±0.008	-	
[H]Heat killed	0.5	-	0.327±0.023	-	
	24	0.002	0.898±0.004*	14.7	
[T] Treated	20	0.005	0.811±0.008*	27.7	
	14	0.01	0.723±0.006*	40.9	0.015
	10	0.02	0.613±0.006*	58.9	
	6	0.04	0.497±0.005*	74.6	
	2	0.08	0.346±0.006*	97.2	

C Positive controls, HNegative controls, TTreated parasite with extract fraction.

*P values correspond to the levels of significance, $P < 0.05$ when compared to the mean value of O.D. observed for the formazan formed for treated and control parasites.

2 to 24hrs incubation respectively but all parasites were active in control (Table 1). The results exhibited that, concentrations of extract fraction, inhibits the motility very fast at concentration dependant manners.

MTT – Formazan colorimetric assay

Antifilarial activity of extract fraction was confirmed by MTT assay. The formazan was extracted in DMSO. 0.327 value obtained for the heat - killed parasite because very less amount of formazan was produced in killed parasite. The, percentage inhibition .(>50%) was 58.9, 74.6 and 97.2% at 0.02, 0.04 and 0.08 mg/mL at 10, 6 and 2hrs incubation, considered significant activity of extract fraction (Table 2). Inhibitory concentration (50%) was calculated to be 0.015 mg/mL.

MDA Estimation

The lipid peroxidation was checked by measuring the MDA levels in parasite culture. It is carried out by TBA test which was modified by Satoh K in1978. The concentrations of MDA were calculated (Table- 3). The absorbance values were plotted on a standard graph The MDA levels for test 0.7, 1.1, 1.7, 2, 2.3 and 2.9 for control 0.2 nM/mL were calculated. MDA values were obtained as antifilarial activity obtained (Figure 1).

Protein carbonilation

The protein carbonilation content in parasite culture after 24 hrs expressed in nM/mg. Carbonyl content 0.09, 0.14, 0.23, 0.39, 0.51, 0.86 and 0.05 for control 0.4 nM/mg (Table- 3) were obtained. Carbonyl content calculated as antifilarial activity obtained (Figure 2).

Nitric oxide Assay

The Nitric oxide levels in culture supernatants were checked. The O.D. values were plotted on standard graph and the values of Nitric oxide levels were measured (Table- 3). It was represented in µM/mL. The Nitric oxide values 0.009, 0.015, 0.027, 0.054, 0.076 and 0.095, for control it was 0.006µM/mL were calculated. Nitric oxide levels were estimated as antifilarial activity obtained. (Figure 3).

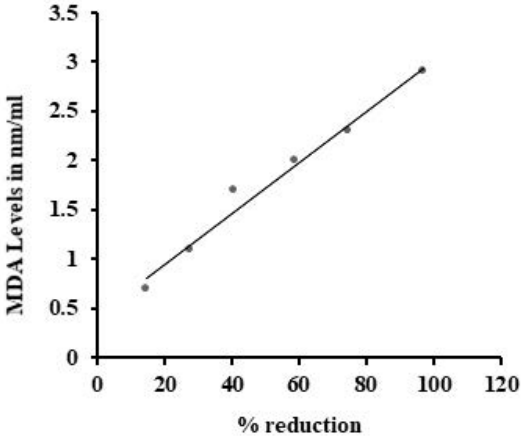

Fig. 1. Levels of MDA after 24 hours extract fraction exposure in the *in vitro* experiments as compared to worms motility. The levels were expressed in nM/mL.

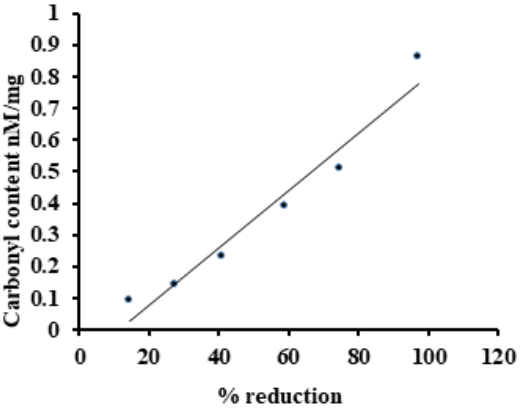

Fig. 2. Levels of Carbonyl content after 24 hours extract fraction exposure in the *in vitro* experiments as compared to worms motility. Carbonyl content were expressed in nM/mg.

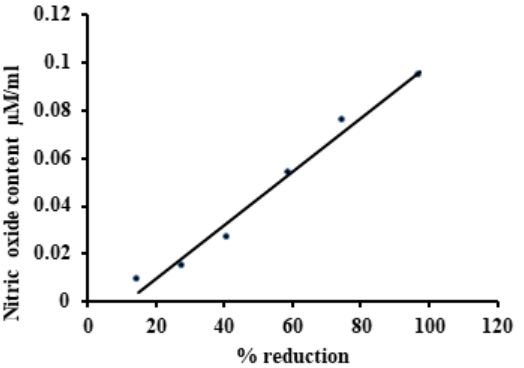

Fig. 3. Nitric oxide levels after 24 hours extract fraction exposure in the *in vitro* experiments as compared to worm motility. Nitric oxide levels were expressed in nM / mL.

Table 3. MDA, Carbonyl content and Nitric oxide levels estimated (nM/mL). Result expressed are Mean ± SEM

Concentrations of extract fraction (mg/mL)	MDA level (nM/mL)	Carbonyl contents (nM/mg)	Nitric oxide level (μM/mL)
0.002	0.7 ± 0.005	0.09 ± 0.012*	0.009 ± 0.001
0.005	1.1 ± 0.152*	0.14 ± 0.019	0.015 ± 0.001*
0.01	1.7 ± 0.173*	0.23 ± 0.045*	0.027 ± 0.004*
0.02	2 ± 0.152*	0.39 ± 0.017*	0.054 ± 0.002*
0.04	2.3 ± 0.115*	0.51 ± 0.035*	0.076 ± 0.001*
0.08	2.9 ± 0.205*	0.86 ± 0.049*	0.095 ± 0.001*
Control	0.2 ± 0.057	0.05 ± 0.024	0.006 ± 0.001

*P value represent the levels of significance, $P < 0.05$ considered significant as compared to control values.

DISCUSSION AND CONCLUSION

Due to the huge social and economic encumber of filariasis in the endemic countries, identification of potent therapeutic novel medicine is necessary, as per WHO direction. Herbal drug are being used by most part of global population in the developing countries. These drugs are safe, compatible and suitable for human being with lesser side effects[33]. *Aegle marmelos* Corr. is a traditional medicinal plant used in many Ayurvedic drugs. Leaves are very useful in the treatment of filariasis. Taking three Bael leaves every day helps both in the prevention and cure of filariasis[34.] In the present investigation fraction isolated from *Aegle marmelos* Corr. leaves Chloroform extract, showed significant anti-filarial activity at their respective concentration also found a direct effect of this compound on the adult parasite in dose dependent manner. The oxidative parameters MDA, Carbonyl content and Nitric oxide content values were obtained increasingly as a reduction in parasite motility were found. The result observed for oxidative stress parameters indicate the close connection of oxidative / nitrosative basis in anti filarial activity. The considerable connection between each parameter and reduction in parasite motility at the concentration range indicate a primary effect of such oxidative damage in the parasite by extract fraction. In a similar study high anti filarial activity at less concentration revealed a considerable relationship with oxidative stress parameters in a respective drug content manner for filarial worms[35, 21]. The close association of Nitric oxide found in host defence and intracellular pathogens in various studies[36, 37]. The results of the present study showed the effect of plant fraction might be as a nitrosative or oxidative stress mediated mechanism.

In conclusion, extract fraction isolated from *Aegle marmelos* Corr. leaves has shown significant antifilarial activity against *Setaria cervi* parasite and probable mechanism identified as oxidative. Some active phytoceutical content, might be responsible for filaricidal activity. These findings indicate the significance of identification of active molecule present in *Aegle marmelos* Corr. leaves to formulate the cost effective potential anti filarial drug candidate to fight filariasis.

ACKNOWLEDGEMENT

Authors are thankful to Dr. Zia-Ul Hasan, Department of Botany, Safia Science College, Bhopal (M.P.) for taxonomical identification of the plant species.

Conflict of interest

Author declares that, there is not conflict of interest.

Funding source

This work was supported by Indian Council of Medical Research (ICMR), Govt. of India, New Delhi.

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