Int Aquat Res (2020) 12:267-278
https://doi.org/10.22034/IAR.2020.1905292.1073

ORIGINAL RESEARCH

Protective effects of selected solvent extracts of *Terminalia arjuna* against environment mediated parasitic *Argulus bengalensis* infection in *Labeo rohita*

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Received: 09 August 2020 / Accepted: 19 November 2020 / Published online: 25 December 2020

Abstract The present study was an attempt to perform functional screening and evaluating the effects of solvent extracts of *Terminalia arjuna* against *Argulus bengalensis*. Solvent extracts were used at 10, 20, 30, 40, and 50 mg/L, at 1, 2, 3, 4 and 5 hours and 12, 24, 36,48 and 60 hours under in-vitro and in-vivo conditions, respectively in moderately parasitically challenged (25-30, *A. bengalensis*/fish) *Labeo rohita* juveniles of 30±1.5 g. The 5% dimethyl sulphoxide was used as negative control (DMSO). The LC_{50} values of solvent extracts for *L. rohita* were 67.67±12.59, 78.13±14.17, 79.12±17.68, 156.47±12.67 and 256.43±8.93 mg/L for *Terminalia arjuna* ethanolic bark extract, *Terminalia arjuna* methanolic bark extract and *Terminalia arjuna* acetone bark extract, respectively, at 60 hours interval. Under in-vitro condition, 100% anti-parasitic efficacy (AE) and minimum therapeutic index (TI) value (1.2) was ascertained by *Terminalia arjuna* ethanolic bark extract at 50 mg/L in 2 hour, and minimum LC_{50} was reported by *Terminalia arjuna* ethanolic bark extract under in-vitro condition (13.14±3.79 mg/L) and maximum by *Terminalia arjuna* acetone bark extract under in-vitro condition (75.8±12.69 mg/L) at 5 hour interval. While, under in-vivo conditions, minimum LC_{50} for immersion and bath treatments was observed with *Terminalia arjuna* ethanolic bark extract (27.92±9.56 mg/L) and TAEBIM (33.6±7.58 mg/L), correspondingly, at 60 hours. The minimum TI was reported in bath treatment of *Terminalia arjuna* ethanolic bark extract (1.1). The RPS was also improved in bath treatment as compare to the immersion treatment. The 100% anti-parasitic activity was observed in bath treatment of *Terminalia arjuna* ethanolic bark extract at 24 hours. The PCA bi-plot explains 79.34% and 14.32% variations for component 1 & 2, respectively. The efficacy of solvent extracts varied significantly in response to concentrations of the extracts and exposure times and toxicity of the extracts (Exposure time*extract *treatment: F=16.12, P= 0.04). The study provides, the evidences for safe and effective application of prospective solvent extracts of *T. arjuna* against *A. bengalensis* in *L. rohita* juveniles, and yield first-hand information on acute toxicity of solvent extract in *L. rohita*.

Keywords Functional screening . TI &LC_{50} . RPS & AE . *Argulus bengalensis* . *Labeo rohita* . Solvent extracts of *Terminalia arjuna*

Introduction

Aquaculture, being the fastest food production sector, leading with 10% annual increment since 1984. Indian major carps (IMCs) species are being practiced in different types of aquaculture systems. Ever increasing population exerts a constant pressure to intensify aquaculture culture practices to suffice the demand of animal protein and ascertain livelihood and nutritional security. Intensification of culture practices includes, high stocking and supplementary feeding , thus, creating a favorable platform to microbes to act upon that in turn to disease outbreaks. The main causative agents for disease occurrence are parasites, bacteria, and fungus. Among the parasites *Argulus* spp., commonly known as fish louse creates a serious havoc and

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imposes a huge economic lose approximately 418.61 US$ / hectare and it accounts approximate 30% to the net profit per hectare per year in carp culture including associated factors like mortality and disease management (Sahoo et al. 2013; Das et al. 2018). *L. rohita* which is considered as important candidate species for Indian aquaculture including six species carp poly culture system. Due to intrinsically sensitive and stressful environment fish including *Labeo rohita* has been reported to be infected by *Argulus* spp. during culture in confinement conditions (Gopalakrishnan 1964; Singhal et al. 1990; Sheila et al. 2002, Shailesh and Sahoo 2010; Sahoo et al. 2013). *Argulus* has cosmopolitan distribution across the globe (Rushton-Mellor 1992), and 129 species of *Argulus* have been described from wild and culture system (William 2008) around the world. Genus *Argulus* is represented by 17 species and one subspecies in India of which 14 species and one subspecies are freshwater inhabitants (DevRoy 2015). *Argulus beneglensis* reported from Bengal by Hora (1943) and Ramakrishna (1951).

Argulus a crustacean parasite, recognized as a causative agent for argulosis in fish. The clinical signs of argulosis include dermal ulceration, physiological stress, immunosuppression reduced fish growth, red lesion on fin origin. Severe argulosis also offers a favorable environment to the secondary infections. The pathogenicity of argulosis has explained in different ways *i.e.* some researcher claim that it is not linked as a causative agent for direct mortality in fish (Hoffman 1977) stated that it has cytoplasmic toxin, injected *via* the pre-oral stylet and secretion and ejection of large amount of toxin result in hemorrhage and physical rupture of the fish tissue. The conventional approach for preventing or reducing the argulosis in fish is through the chemical such as avermectin, doramectin, ivermectin, teflubenzuron, deltamethrin, pyrethrin, malathion, cypermethrin and chlorophenol (Treves-Brown 1999; Toovey and Lyndon 2000; Hemaprasanth et al. 2012). In the long term, the constant application of these obnoxious chemicals results in bioaccumulation issues and chemical-specific resistant in *Argulus* spp. (Jones et al. 1992). The efficacy of some of the chemicals such as chloroform and formalin is not up to the mark in terms of imposing unwanted toxicity on the species and environment also (Goven et al. 1980; Klinger and Floyd 2002). These circumstances expel researcher to find out effective, less toxic, easily available and cheaper drug formulation based herbal material and phytochemicals. The application of herbal medicament gaining importance due to twofold reasons, Firstly, the application of herbal medicine has its center stage and expected to increase in the forthcoming future due to their availability and cheap investment also. Secondly, herbal treatments are potentially used in complementary medicine for treatment of various chronic ailments conditions, and could yield an intuitive feeling that "naturalness" is a guarantee of harmlessness (Khodadadi et al. 2011). As per the WHO (2002), approximately 80% of the world's population depend on traditional medicine, and in India 60% of the people in rural areas use herbal medicines. During the last few years, the use of herbal supplements increased from 2.5% to 12% (Stickel and Schuppan 2017).

Fig. 1 *Argulus bengalensis.* The specimen was confirmed by morphological and using identification key for its biological origin

Various herbal products and parts of the plant have been used to cure *Argulus* infected fish species. For instance, azadirachtin used against *Argulus* spp. in goldfish (Kumar et al. 2012a, b; Banerjee and Saha 2013). Similarly, Kumar et al. (2013) evaluated anti-parasitic efficacy of piperine against *Argulus* spp. on *Carassius auratus* under *in-vivo* and *in-vitro* conditions, and rotenone and nicotine have also been applied in *Labeo rohita* (Banerjee and Saha 2013).

Other plant such as *Terminalia arjuna* (Family: Combretaceae) comprising approximately 200 species around the globe and 24 species in India, has also been reported to possess ethnomedicinal properties including antifungal, antibacterial and anti-parasitic (Dwivedi and Chopra 2014; Amalraj and Gopi 2017) and easily available at less price in market. However, it has not been tried for controlling argulosis in fish. Also, the mode of pathogenicity of the argulosis is still unclear, and depends on many factors and associated with prevalence, intensity and prior exposure of the hosts to *Argulus* infection (Taylor et al. 2006). Despite of numerous ethnomedicinal importance of *T. arjuna*, no previous study has attempted to correlate the anti-parasitic activities of solvent extracts of *T. arjuna* as natural anti-parasitic agents with their cytotoxicity and estimation of their therapeutic indices, RPS AE, EC_{50} and LC_{50} values. Therefore, it is highly mandatory to screen the functionality of the plant material for evaluating mode of action against argulosis. In recent past, studies carried out as bath treatment of herbal products against *Argulus* infected fish but immersion treatment with herbal extracts for long term impact and sustainability has not been executed. Such comparative studies aimed to provide scientifically-based evidence of the therapeutic safety margins of agents which may be administered in the treatment of a disease. Before becoming a drug for therapeutic use, a medicinal agent, either synthetic or of herbal origin, vigorous in vitro and in vivo studies are needed to determine its safety and efficacy. In these backdrops, the present study was undertaken to evaluate the therapeutic dynamics and functional efficacy of selected solvent extracts of an ethnomedicinal plant, *T. arjuna* for controlling argulosis in freshwater fish, *Labeo rohita*.

Materials and methods

In-vitro study on efficacy of selected solvent extracts

The preparation of solvent extracts has already been published by Meena et al. (2020a). Briefly, 100g dry powder of three parts (leaf, bark and fruit) of *Terminalia arjuna* plant material was taken into seven solvents based on their polarity starting form hexane, ethyl acetate, chloroform acetone, ethanol, methanol and distilled water (1:5), and kept for 36 hrs in shaking incubator. Thereafter, it was filtered through Whatman No. 1(40 mm) filter and solvent was evaporated in rotary evaporator till it reached 1/10 of its volume. Then, solvent extract was dried and yield % was recorded as follows ethanol extract (23.6±0.026), methanol extract (22.23±0.017) and acetone extract (11.36±0.005). These 21 solvent extracts of *T. arjuna* were used for the screen of their efficacy against *A. bengalensis,* applying negative and positive control for initial screening purposes, and thereafter best five were selected for further studies. The working concentration was prepared by dissolving extracts from mother dried stock to dimethyl sulphoxide (DMSO) to achieve five concentration *viz.* 10 mg/l, 20 mg/l, 30 mg/l, 40 mg/l, and 50 mg/l for *in-vitro* study. Meena et al. (2020b) has already done the toxicity study of the 21 solvent extract and also elucidated the toxicity of the solvent for ensuring safety and efficacy of herbal extract only. Briefly, 50 *Argulus bengalensis* with the help of dropper were taken into 50 ml of Dw (distilled water) in 100 ml capacity of sample container. To this 10 ml of the DMSO prepared extract was added and the mortality of the *Argulus* at different time intervals was noted and the mortality of *Argulus* was confirmed by seeing the movement of the specimen under the microscope.

Estimation of LC_{50} of solvent extracts against *L. rohita* under in-vivo and *Argulus bengalensis* under in-vivo and in-vitro experiments

For *in-vitro* experiment 100, *A. benegalens* being stocked in glass jar containing 100 ml water and to this 10 ml of extract was added to each jar from DMSO stocked solution of 10-50 ppm. The numbers of dead *Argulus* after 1, 2, 3, 4 and 5 hrs was noted. For conducting *in-vivo* experiment (bath and immersion) *L. rohita* was made infected with *A. bengalensis* by co-habitation method under regular supervision for a

duration of 10 days. Upon moderate infection (25-30 argulus / fish), 6 fish were gently picked up and transferred in triplicate manner in 500 L capacity plastic tanks with 100 L water. For immersion treatment, each tank designated as per the concentration ranges from 10-50 ppm, 100 ml extract from DMSO mother stock was added and argulus mortality was observed at regular intervals for 12, 24, 26, 48 and 60 hrs. For bath treatment they fishes were being given bath treatment for 3 consecutive days in 5 separate treatment jar of having 5 L water with 1000 ml DMSO extract from each concentration (10-50 ppm) and exposed for 5 minutes each day. Subsequently, after bath fish were kept in 5 different tank designated as per the bath treatment (10-50 ppm) and kept under observation at regular intervals 12, 24, 36, 48 and 60 hrs. For each treatment dead *A. bengalensis* were noted using manual counting of attached *A. bengalensis* on fish surface particularly caudal peduncle and pectoral fin, around the gill region and haemorrhagic sites. The LC_{50} was calculated as per the Finney (1952) with slight modification. Simultaneously, LC_{50} of solvent extracts against *L. rohita* was also estimated following the method of Finney (1952). Briefly, *in-vivo* study was conducted for assessing the toxicological study of the solvent extracts of Arjuna. A total of 20 fish (avg. wt. 30±1.5 g) were kept for acclimatization and then transferred to each 500 L capacity plastic tanks (designated as 10, 20, 30 40 50 ppm) in triplicate and static conditions was maintained with proper aeration facility. The number of dead fish was noted against concentration were noted at 12, 24, 26, 48 and 60 hrs intervals and LC_{50} was calculated (Finney 1952).

Estimation of relative percentage survival (RPS) of *L. rohita* and anti-parasitic efficacy (AE) percentage of solvent extracts against *Argulus bengalensis*

It is an indication of the potential of herbal extract and can be calculated as follows (Wang et al. 2009):

$$AE\ (\%) = (B-T)/B*100$$

Where, B= mean *A. bengalensis* survived in control, control includes 5% DMSO as negative control; T= mean *A. bengalensis* survived in treatment.The RPS under in-vivo (Bath and immersion) treatment for infected *L. rohita* upon the treatments was calculated as follows:

$$RPS = (1- \%\ \text{mortality in treated fish}) \times 100)/ \%\ \text{mortality in control}$$

Therapeutic Index

After calculating the LC_{50} at particular time and dose, the relationship of dose-response is used to determine the therapeutic index which can be calculated by the following formula:

$$TI = LC_{50}/EC_{50}$$

LC_{50}= mean lethal concentration; EC_{50}= mean effective concentration

EC_{50} is the effective concentration of a drug, extract or other bioactive principle that asceratin100% mortality of the parasites at particular time intervals. EC_{50} is equal or less than the LC50 value for an effective bioactive compound and vice-versa. For its apparent calculation, in excel at different time interval as per the concentration a dose response curve is made, the 100% mortality or 0% survival ensure predicted EC_{50} accordance to its maximal responses.

Significance of interactions among time, extract, factors and solvent extracts and establishment of correlation matrix and bi-plot between solvent extract and parameters of toxicity efficacy

The interactions between and among the parameters were established deploying three-way ANOVA in SPSS ver. 20, correlation matrix and bi-plot was constructed using PAST4.03 (Hammer et al. 2001) and Minitab 18 software. Images were edited using Paint 3D ver.16.

Results

In-vitro screening of selected solvent extracts and efficacy study

The *in-vitro* screening of the herbal extracts includes initial screening based on LC_{50} values of 21 solvent extracts comprising 7 solvent extracts of each part *i.e.* leaf, fruit and bark as mentioned earlier. Out of 21, best five solvent extracts were. *Terminalia arjuna* acetone fruit extract, *Terminalia arjuna* ethanol bark extract, *Terminalia arjuna* methanol bark extract, and *Terminalia arjuna* methanol fruit extract. These solvent extracts further used for studying other parameters such as TI, RPS and AE of in-vitro and in-vivo experiments.

Estimation of LC_{50} of solvent extracts against *L. rohita* under in-vivo and *Argulus bengalensis* under in-vivo and in-vitro experiments

The result of in-vivo toxicity study of five solvent extract of *T. arjuna* showed that TAAB showed least toxicity as indicated by LC_{50} value (854.12 mg/L) followed by TAMF (442.56 mg/L), TAAF (288.35 mg/L), TAMB (228.43 mg/L) and TAEB (213.21 mg/L) at 12 hour time interval while minimum values were reported at 60 hour time interval as 67.67, 78.13, 79.12, 156.47 and 256.43 mg/L for TAEB,TAMB,TAAF, TAMF and TAAB, respectively (Table 1). For all five extracts, LC_{50} value was decreasing as time of exposure increases and maximum LC_{50} was observed in TAABIN (218.93 mg/L) at 1 hour interval and LC_{50} at 5 hour interval was minimum for each solvent extract and reported least in TAEBIN (19.14mg/L) and maximum in TAABIN (22.56. 8mg/L).

Table 1 Showing LC50 values of effective solvent extracts against *L. rohita* and against Argulus. N=3, values are represented as mean ± standard error

Name of Treatment	**Solvent concentration**	**10-50 mg/L**	**10-50 mg/L**	**10-50 mg/L**	**10-50 mg/L**	**10-50 mg/L**
In-vitro treatment (IN)	**In-vitro treatment (IN)**	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**5 hr**
	Control (DMSO ml/L)	1560.23±12.45	1293±13.65	1068±15.64	989±16.18	876±15.87
	TAABIN	218.93±8.45	163.21±9.38	100.62±11.83	88.55± 9.78	75.8±12.69
	TAAFIN	129.04±7.67	100.67±10.45	54.06±6.98	39.05±9.37	34.96±6.79
	TAEBIN	80.51±11.23	28.55±4.59	16.31±4.13	14.9±3.68	13.14±3.79
	TAMBIN	123.15±12.56	78.85±8.45	31.05±6.64	25.07±2.67	22.55±2.69
	TAMFIN	133.49±12.24	109.23±6.78	80.39±7.34	59.5±6.49	43.11±5.98
In-vivo (Immersion), IM	**Solvent extracts /LC-50 at time interval**	**12 hr**	**24 hr**	**36 hr**	**48 hr**	**60 hr**
	Control (DMSO ml/L)	2255.23±22.45	2089±18.69	1768±15.64	1489±16.18	1176±15.87
	TAABIM	554.72±9.53	342.23±9.87	290.92±11.23	233.49±9.76	165.79±7.29
	TAAFIM	164.21±8.91	117.96±7.84	67.48±8.97	52.65±8.48	34.37±8.95
	TAEBIM	107.41±5.46	94.51±5.72	62.17±4.67	45.06±5.27	33.6±7.58
	TAMBIM	125.82±9.49	99.4±7.23	77.96±6.28	57.32±7.34	50.77±5.89
	TAMFIM	342.89±11.28	237.08±9.47	137.74±7.78	122.85±8.37	83.48±5.94
In-vivo (bath), BA	**solvent extracts/ LC-50 at time interval**	**12 hr**	**24 hr**	**36 hr**	**48 hr**	**60 hr**
	Control (DMSO ml/L)	1967±24±45	1745.34±37	1546.67±12.56	1342±15.56	1080.23±12.67
	TAABBA	359.72±10.23	232.34±12.26	136.599±13.47	63.74±16.12	49.48±17.65
	TAAFBA	119.22±12.56	107.49±14.23	57.07±9.34	53.18±13.23	41.75±12.27
	TAEBBA	95.28±9.45	79.89±6.89	57.57±8.95	53.32±7.34	27.92±9.56
	TAMBBA	115.12±8.25	97.89±6.78	68.35±7.34	46.47±7.28	36.33±7.45
	TAMFBA	229.26±6.78	196.11±7.98	112.76±6.24	51.57±8.58	44.36±8.23
In-vivo (Fish)	**solvent extracts/ LC-50 at time interval**	**12 hr**	**24 hr**	**36 hr**	**48 hr**	**60 hr**
	TAABBA	854.12±15.56	482.21±6.78	382.22±7.89	287.12±7.23	265.43±8.93
	TAAFBA	288.35±6.28	249.55±8.95	187.48±9.67	138.52±13.28	79.12±17.68
	TAEBBA	213.21±9.68	168.53±11.45	136.67±15.67	113.07±14.67	67.67±12.59
	TAMBBA	228.43±14.27	186.42±15.83	165.24±13.85	145.34±13.96	78.13±14.17
	TAMFBA	442.56±13.78	357.28±15.78	263.34±14.58	222.34±13.28	156.47±12.67

Estimation of Relative percentage survival (RPS) of *L. rohita* and anti-parasitic efficacy (AE) percentage of solvent extracts against *Argulus bengalensis.*

Under in-vivo condition, the AE in bath treatment was found to be higher at 36, 48 and 60 hours intervals for TAEB50BA, at 48 and 60 hour for TAAF50BA, TAMB50BA and TAMF50BA. The solvent extracts at different time interval and concentration showed a diverse pattern of distribution of AE *i.e.* 100 % AE was showed by TAEB 40 at 4 &5 hours; TAAF 40 at 5 hour; TAMB 40 at 5hour; TAAB 50 at 5hour; TAAF 50 at 4 & 5 hours; TAEB 50 at 2, 3, 4& 5 hours; TAMB 50 at 4 &5 hours and TAMF 50 at 5 hour (Table 2). The LC_{50} value of each extract in immersion study was higher side as compared to the bath treatment (Table 2). The trend of LC_{50} values for both treatments at every time interval following the order as TAAB>TAMF>TAAF> TAMB>TAEB. Minimum LC_{50} values in immersion and bath treatments was observed in TAEBBA (27.92 mg/L) and TAEBIM (33.6 mg/L) at 60 hour interval while maximum LC_{50} was reported in TAABIM (554.72 mg/L) and TAABBA(359.52 mg/L) at 12 hour interval.

Comparative RPS of *Labeo rohita* under *in-vivo* study showed that in bath treatment RPS varied from >65 %-≥ 90% and in immersion treatment, it was approximately ranged between 50% - 85%. TAAF40 BA (60 hour), TAEB50 BA (48 hour) and TAMF 50 BA (60 hour) showed maximum RPS (≥ 80%-90%) in bath treatment while in the case of immersion it was maximum in TAEB 50 IM (85%) at 60 hour followed by TAMB50 IM (≤ 80%) at 60 hour TAMB50 BA, TAEB50 BA and TAAF50 BA exhibited the RPS overlapping at 12, 24 & 36 hour time interval whereas TAAMB40 IM, TAEB40 IM, TAAF 30 IM etc., were found to overlap at 12 and 24 hours interval (Table 2).

Estimation of TI

The TI values of in-vivo and in-vitro treatments are given in Fig. 2. TI value is an index of efficacy of the solvent extracts against treated material and exhibits and inverse relationship with LC_{50} of the corresponding solvent extracts. Maximum TI value was exhibited by TAABIM (4.6)>, TAMFIM (3.9) > and TAAB (3.3).

Interactions among time, extract, factors and solvent extracts and establishment of correlation between solvent extract and parameters of toxicity efficacy

Interactions between solvent extracts found to be highly significant (P <0.001), interactions among treatments was also be found significant (P<0.004), impact of time*treatment were also significant (P<0.03), time * extract have significant interaction (0.02), extract*treatment (P <0.01) and time*extract*treatment

Fig. 2 Comparative TI value for the corresponding solvent extracts against *A. bengalensis*

showed significant interactions (P <0.04). The F- value exhibited inverse relationship with *P- value* and the decreasing trend for *F value* is as follows; Extract (4453.21) > treatment (2141.57) > extract*treatment (735.01) > time *extract (364.73) > time*treatment (58.25) and time*extract*treatment (16.12) (Table 3). PCA 1 and PCA 2 explain 79.34% and 14.32% variations, respectively. LC_{50} negatively correlated with PE and TAABBA has a positive correlation with LC_{50} whereas PE is positively correlated with TAAFBA>TAEBIM> TAMBIM>TAAFIM.RPS is negatively correlated with TI and RPS has a positive

Table 2 AE and RPS values of *in-vivo* and *in-vitro* study of solvent extracts of *T. arjuna* against *A. bengalensis*

	Relative Percentage Survival Survival (RPS) of *L. rohita*					Mean Surviving *A. bengalensis*					Anti-parasitic efficacy percentage				
Solvent Extract/time	**12hr**	**24hr**	**36hr**	**48hr**	**60hr**	**12hr**	**24hr**	**36hr**	**48hr**	**60hr**	**12hr**	**24hr**	**36hr**	**48hr**	**60hr**
Control BA	55.4	54.7	54.7	54.7	53.6	100	95	97	93	87	0	0	0	0	0
TAAB10BA	66.7	66.7	66.7	66.7	66.7	99	95	93	88	79	1	5	7	12	21
TAAF10BA	66.7	70.0	73.3	76.7	80.0	94	87	82	75	69	6	13	18	25	31
TAEB10BA	70.0	73.3	76.7	80.0	83.3	91	83	74	69	63	9	17	26	31	37
TAMB10BA	66.7	70.0	73.3	76.7	80.0	93	85	77	72	68	7	15	23	28	32
TAMF10BA	66.7	66.7	70.0	70.0	73.3	96	91	87	81	74	4	9	13	19	26
TAAB20BA	70.0	70.0	73.3	73.3	73.3	92	83	79	74	69	8	12	17	20.20	24.49
TAAF20BA	73.3	73.3	73.3	80.0	80.0	82	56	54	43	38	18	32	44	45.45	56.12
TAEB20BA	76.7	76.7	76.7	80.0	83.3	76	47	41	33	29	24	40	53	58.59	66.33
TAMB20BA	73.3	73.3	73.3	80.0	80.0	79	54	44	38	34	21	36	46	55.56	61.22
TAMF20BA	70.0	70.0	73.3	73.3	76.7	88	66	64	53	48	12	27	34	35.35	45.92
TAAB30BA	70.0	73.3	76.7	76.7	76.7	87	81	78	72	69	13	19	21.21	27.27	29.59
TAAF30BA	76.7	76.7	80.0	80.0	80.0	68	55	45	34	24	32	45	54.55	65.66	75.51
TAEB30BA	80.0	83.3	83.3	83.3	86.7	54	45	32	24	13	46	55	67.68	75.76	86.73
TAMB30BA	76.7	80.0	83.3	83.3	86.7	63	48	36	28	17	37	52	63.64	71.72	82.65
TAMF30BA	70.0	73.3	76.7	76.7	76.7	78	63	56	44	33	22	37	43.43	55.56	66.33
TAAB40BA	73.3	76.7	80.0	80.0	80.0	77	71	68	62	59	23	28.28	30.61	36.73	39.18
TAAF40BA	80.0	80.0	83.3	83.3	83.3	46	33	17	9	0	54	66.67	82.65	90.82	100.00
TAEB40BA	83.3	83.3	86.7	86.7	90.0	38	24	6	0	0	62	75.76	93.88	100.00	100.00
TAMB40BA	80.0	80.0	83.3	83.3	86.7	42	29	13	4	0	58	70.71	86.73	95.92	100.00
TAMF40BA	73.3	76.7	80.0	83.3	83.3	52	42	22	17	6	48	57.58	77.55	82.65	93.81
TAAB50BA	76.7	80.0	80.0	83.3	83.3	57	41	38	32	23	43	58.59	61.22	67.35	70.10
TAAF50BA	83.3	83.3	83.3	86.7	86.7	20	15	0	0	0	80	84.85	100	100	100
TAEB50BA	83.3	83.3	86.7	90.0	90.0	8	0	0	0	0	92	100	100	100	100
TAMB50BA	83.3	83.3	83.3	86.7	90.0	14	8	0	0	0	86	91.92	100	100	100
TAMF50BA	76.7	80.0	83.3	86.7	86.7	36	19	4	0	0	64	80.81	95.92	100	100
Control IM	51.5	52.2	50.6	50.3	49.8	100	97	95	92	85	0	0	0	0	0
TAAB10IM	50.0	50.0	53.3	53.3	53.3	99	96	94	90	82	1	4	6	10	18
TAAF10IM	50.0	50.0	56.7	60.0	60.0	95	89	85	78	71	5	11	15	22	29
TAEB10IM	56.7	56.7	60.0	63.3	66.7	92	85	77	71	65	8	15	23	29	35
TAMB10IM	53.3	53.3	53.3	60.0	60.0	94	88	81	76	70	6	12	19	24	30
TAMF10IM	50.0	50.0	53.3	56.7	60.0	97	93	91	85	79	3	7	9	15	21
TAAB20IM	50.0	53.3	56.7	56.7	60.0	94	86	81	77	72	6	14	17.18	19.43	24.53
TAAF20IM	53.3	53.3	56.7	63.3	63.3	84	65	59	52	43	16	10	13.00	18.18	20.41
TAEB20IM	56.7	56.7	63.3	63.3	66.7	79	61	49	41	36	21	39	49.51	56.16	61.27
TAMB20IM	53.3	53.3	56.7	60.0	60.0	82	63	53	45	41	18	37	45.46	52.08	56.16
TAMF20IM	50.0	53.3	56.7	56.7	60.0	86	71	66	58	51	14	29	32.33	38.82	45.96
TAAB30IM	56.7	56.7	60.0	63.3	66.7	90	83	81	75	71	10	17	17.18	23.24	25.55
TAAF30IM	56.7	60.0	60.0	66.7	66.7	72	60	52	41	32	28	19	24.24	30.30	36.73
TAEB30IM	60.0	66.7	70.0	73.3	73.3	59	51	41	32	18	41	49	57.59	66.68	79.63
TAMB30IM	56.7	60.0	63.3	70.0	73.3	70	55	42	34	21	30	45	56.58	64.66	76.57
TAMF30IM	56.7	56.7	60.0	63.3	66.7	82	68	62	53	40	18	32	36.37	45.46	57.18
TAAB40IM	56.7	60.0	63.3	66.7	66.7	82	75	71	68	60	18	23.24	25.55	28.61	35.14
TAAF40IM	56.7	63.3	63.3	66.7	70.0	51	38	24	21	7	49	60.62	73.51	76.57	89.78
TAEB40IM	63.3	70.0	70.0	76.7	80.0	41	31	18	14	0	59	67.69	79.63	83.71	100
TAMB40IM	60.0	60.0	66.7	70.0	76.7	44	34	23	17	0	56	64.66	74.53	80.65	100
TAMF40IM	56.7	60.0	63.3	66.7	70.0	56	47	29	24	13	44	51.53	68.41	73.51	83.60
TAAB50IM	56.7	60.0	66.7	70.0	73.3	61	47	42	25	17	39	51.53	55.14	62.29	62.98
TAAF50IM	63.3	66.7	70.0	76.7	80.0	32	25	11	7	4	68	73.75	86.78	92.90	97
TAEB50IM	66.7	73.3	76.7	80.0	86.7	24	13	0	0	0	76	85.87	100	100	100
TAMB50IM	63.3	66.7	70.0	76.7	83.3	29	21	9	0	0	71	77.79	88.82	100	100
TAMF50IM	63.3	66.7	70.0	73.3	76.7	41	23	14	0	0	59	75.77	83.71	100	100
Solvent Extract/time	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**5 hr**
Control IN	NA	NA	NA	NA	NA	99	96	93	89	83	0	0	0	0	0
TAAB10IN	NA	NA	NA	NA	NA	94	88	82	76	70	6	12	16.33	22.45	28.57
TAAF10IN	NA	NA	NA	NA	NA	86	80	72	62	52	14	20	26.53	36.73	46.94
TAEB10IN	NA	NA	NA	NA	NA	80	68	56	42	32	20	32	42.86	57.14	67.35
TAMB10IN	NA	NA	NA	NA	NA	84	76	66	50	44	16	24	32.65	48.98	55.10
TAMF10IN	NA	NA	NA	NA	NA	88	84	76	70	62	12	16	22.45	28.57	36.73
TAAB20IN	NA	NA	NA	NA	NA	90	84	76	66	60	10	14.29	22.45	31.25	37.50
TAAF20IN	NA	NA	NA	NA	NA	82	72	64	56	46	18	26.53	34.69	41.67	52.08
TAEB20IN	NA	NA	NA	NA	NA	70	60	50	32	20	30	38.78	48.98	66.67	79.17
TAMB20IN	NA	NA	NA	NA	NA	76	66	54	38	30	24	32.65	44.90	60.42	68.75
TAMF20IN	NA	NA	NA	NA	NA	86	78	72	64	54	14	20.41	26.53	33.33	43.75
TAAB30IN	NA	NA	NA	NA	NA	78	66	58	52	46	22	32.65	40.82	46.94	53.06
TAAF30IN	NA	NA	NA	NA	NA	64	50	42	34	22	36	48.98	57.14	65.31	77.55
TAEB30IN	NA	NA	NA	NA	NA	54	42	32	10	0	46	57.14	67.35	89.80	100
TAMB30IN	NA	NA	NA	NA	NA	60	52	38	18	6	40	46.94	61.22	81.63	93.88
TAMF30IN	NA	NA	NA	NA	NA	70	58	50	42	28	30	40.82	48.98	57.14	71.43
TAAB40IN	NA	NA	NA	NA	NA	62	54	44	34	22	38	44.90	55.10	65.31	77.55
TAAF40IN	NA	NA	NA	NA	NA	52	42	34	14	0	48	57.14	65.31	85.71	100
TAEB40IN	NA	NA	NA	NA	NA	36	22	18	0	0	64	77.55	81.63	100	100
TAMB40IN	NA	NA	NA	NA	NA	42	30	26	0	0	58	69.39	73.47	100	100
TAMF40IN	NA	NA	NA	NA	NA	58	50	38	26	6	42	48.98	61.22	73.47	93.88
TAAB50IN	NA	NA	NA	NA	NA	46	34	24	14	0	54	65.31	75.51	85.71	100
TAAF50IN	NA	NA	NA	NA	NA	32	22	14	0	0	68	77.55	85.71	100	100
TAEB50IN	NA	NA	NA	NA	NA	16	0	0	0	0	84	100	100	100	100
TAMB50IN	NA	NA	NA	NA	NA	22	10	6	0	0	78	89.80	93.88	100	100
TAMF50IN	NA	NA	NA	NA	NA	38	30	18	6	0	62	69.39	81.63	93.88	100

Table 3 Significance of interactions among the factors

Factor	Mean Square	F-value	P-value
Extract	169066.7	4453.21	0.0001
Treatment	81305.04	2141.57	0.004
Time * Extract	13846.91	364.73	0.02
Time * Treatment	2211.58	58.25	0.03
Extract * Treatment	27904.79	735.01	0.01
Time*Extract * Treatment	611.912	16.12	0.04

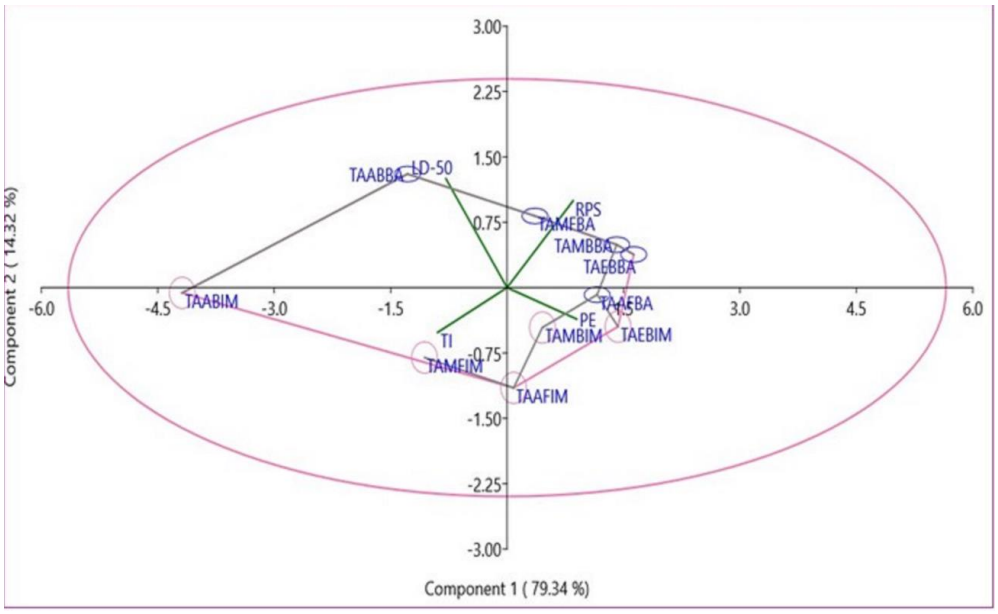

Fig. 3 Showing correlation between solvent extracts and efficacy parameters

correlation with TAEBBA> TAMBBA>TAMFBA whereas TI has a positive correlation with TAMFIM and TAABIM (Fig. 3).

Discussion

In the present experiment, the functional screening in terms of *in-vitro* study for selecting the most effective solvent extracts against *A.bengalensis* was done. Also the *in-vivo* study (bath and immersion treatments) for optimizing the effective solvent extracts and their dose in both the treatments was carried out. The *Argulus* species used in the present study was identified as *A. bengalensis* based on morphological characteristics of the abdomen, cephalothoracic region and coxal spines which resemblance with the description recorded by previous researcher (Hora 1943; Ramkrishana 1951; Fryer 1968, 1982; Rushton-Mellor 1994; Noaman et al. 2010; Wafer et al. 2015). The *Argulus* infestation in the present study was achieved through co-habitation method and it was a medium infestation 25-30 *A. bengalensis*/ fish. More or less similar infection intensity was also found by Kumar et al. (2012b) in experimentally challenged gold fish (15-20 parasites/ fish). Gonzalez et al. (2018) also reported a significantly high prevalence of *Argulus* parasite during the summer season. The successful infection of *A. japonicus* to healthy *C. auratus* through co-habitation method showed an average intensity of 20 to 26 juvenile *Argulus*/ fish. The present study showed the dose-dependent responses of the moderately infected *Labeo rohita* juveniles which is in accordance to the studies of (Kumar et al. 2012b) who claimed that at higher concentration, piperine solution was found to be more effective as compare to lower concentration to eliminate ectoparasite *Argulus* spp. and its effectiveness was found to be dose dependent. The *in-vitro* study revealed best five solvent extracts *i.e.* TAEB, TAAB, TAMB, TAMF and TAAF and their LC_{50} values of these five extracts at all-time intervals followed the same trend as TAAB>TAMF>TAAF>TAMB>TAEB that might be attributable to their toxicological efficacy

against *Argulus* spp., under in-vitro conditions which is supported by the studies of (Kumar et al. 2012a) who explained the more efficacy of herbal material in in-vitro conditions. Further, TI of the solvent extracts was found to be proportionately related with LC_{50} which indicates the potential of the particular solvent extract against *A. bengalensis* infestation. Kumari et al. (2019) also revealed that as LC_{50} decreases, the exposure time increases, and however, unlike the study of (Kumari et al. 2019), the higher TI value not recorded in the present study for the most efficient solvent extract. This deviation might be attributed due to two factors, EC_{50} and LC_{50}. Comparatively *Terminalia arjuna* ethanol bark extract out of five solvent extracts *i.e.* Terminalia arjuna methanol bark extract *Terminalia arjuna* acetone fruit extract *and Terminalia arjuna acetone bark extract in all treatments including in-vitro and in-vivo* (bath and immersion) showed the highest efficacy in terms of lower LC_{50} and TI values and Higher RPS. The highest efficacy might be archived to the presence of phenolic and other bioactive compounds that is in harmony to the previous studies (Mordue and Nisbet 2000; Costa et al. 2008; Kumari et al. 2019) where it was pointed that out of all solvent extract organic solvent extract such as Ethanol exhibited maximum efficacy against *Argulus* spp. The higher efficacy of ethanol barks extract used in present study might be due to presence of terpenoid in ethanolic and aqueous extracts, which was lacking or in less amount in the methanol, chloroform and acetone extracts. It can be publicized that LC_{50} decreases with increase of exposure time and AE proportionately increases that is in harmony to the previous studies (Kumar et al. 2012a, 2012b; Kumari et al. 2019).

In the present study, the LC_{50} values of solvent extracts, particularly, serial fraction of 100% ethanolic bark extract, for fish were higher than the ED_{50} and LC_{50} values of the solvent extracts for *A. bengalensis*. Contrary to the present study, Suely et al. (2016) reported the LC_{50} for 80% ethanolic bark extract against freshwater catfish, *Heteropneustes fossilis*, while (Mishra et al. 2005 and Srivastava et al. 1995) claimed that 96-h LC_{50} value of this plant extract was found to be lower than the effective doses of two synthetic chemicals such as malachite green and cypermethrin studied against the same catfish, and deviation in value was reportedly, 5.6 and 7.2 mg/L, respectively. The huge deviation in the finding of the present study, from the earlier reports might be due to difference in extraction method mode of treatment, the season of collection of herbal material, water quality parameters and location of the sample collection which supported by the findings of (Chandrawathani et al. 2002). In the present experiment, the LC_{50} values, for both bath treatment and immersion follow the same trend for each extract at different time interval and bath treatment exhibited the lower LC_{50} values as compared to the immersion treatment. In accordance to present study, Kumar et al. (2012b) and Kumari et al. (2019), also highlighted the efficacy of bath treatment as fast relief from *Argulus* load in goldfish. TI also followed the same fashion as in the case of LC_{50} for both the treatments whereas RPS and AE followed a diverse pattern of efficacy that is supported by (Kumar et al. 2012b). The toxicological study of two factors, LC_{50} and EC_{50} play an important for ensuring the drug efficacy and safety concern for the host animals. In the present study the LC_{50} values for fish was recorded much higher than the LC_{50} value for all treatments (*in-vitro*, & *in-vivo* including bath and immersion) and also the EC_{50} for each solvent extract is either much below or just less than the LC_{50} values which indicates that the solvent which having vast difference in these two parameters, LC_{50} and EC_{50} would definitely be safe for host but may not be much effective as compare to the solvent extract those which having less difference between these two and vice-versa that corollary to the findings of (Kumar et al. 2012b) who has explained that their extracts ensure the safety of the host organism, and as the median EC_{50} was nearly four times less than the toxic dose, this indicates that it has no risk for the host. Similarly, the trend for all parameters was more or less same in each treatment, however, the significant differences between the parameters such as LC_{50}, PRS, TI and AE % might be due to temporal difference in the intensity and occurrence of parasites and also the fact that in in-vitro condition the parasite does not has the host and attachment to the surface and the handling during bath treatment may likely to provoke the short and acute exposure to the parasite which might be enhancing the efficiency of the solvent extracts that corroborate with the previous studies (Kirby 1996; Kumar et al. 2012b; Mamadou et al. 2013) who explained differences in efficacy under in vitro and in vivo test with drugs and *Ocimum gratissimum* extract against *Argulus* spp. In any system, water quality is considered one of the most important factors for its flora and fauna (Zabed et al. 2014). In our experiment, most of the parameters were found in optimum range except temporal alterations in DO and pH and static variation in ammonia which might be due to extraneous metabolic activities taking place during the exposure period. Moreover, under in-vivo condition other extrinsic factors also influence the infestation intensity such as alteration in water quality parameters particularly, DO and

pH due to herbal extract during the exposure time that is supported with by the past studies (Binh 2016; Mondal et al. 2007) who explained the variation in water certain water quality parameters such as ammonia, free carbon dioxide, nitrite, nitrate, and phosphate in the extract-exposed tanks reported excess due to addition of extract and higher activity of exposed fish including excessive mucus production as found when bio-pesticides like nimbecidine and neem gold applied in the fish pond. Similarly, (Srivastava et al. 1995) illustrated that DO depletion in bio-pesticides exposed tank related to application of herbal extract or synthetic toxicant added to water which causes a considerable fluctuation in different quality parameters and consequently, fishes have encountered a hypoxic condition (Winkaler et al. 2007). Having compared, the all toxicological and herbal efficacy parameters of immersion and bath treatment, it can be illustrated that bath treatment could yield fast response in reducing the intensity of infection of *A. bengalensis* while immersion treatment was responding slower comparatively. From the glance of Table 1, it can be synthesized that in-vitro treatment, in vivo treatment could result in combined significant effective control of *Argulu bengalensis,* as per the nature of solvent extracts, timing of exposure, mode of application, dose of solvent extracts and treatment conditions that indicate that for effective control of *A. bengalensis* is depending on these factor up to a great extent. Similarly, higher F value (4453.21) with low P (0.0001) value validates the efficacy of solvent extract for controlling the *A. bengalensis* in *Labeo rohita* juveniles that is in agreement with Suely et al. (2016) who found that percent mortality of fishes varied significantly in response to concentrations of the extract and exposure times (between exposure time $F= 36.57$, $P <0.001$; between concentrations $F= 39.93$, $P <0.001$). After analyzing the Fig. 2, it can be suggested that solvent extracts such as TAABIM and TAMFIM and TAABBA having higher TI values can be considered safe for host in immersion and bath treatment, respectively, while, other solvent extracts are having twofold benefits, safe for host and can be a potential material for drug synthesis against *A. bengalensis*. Similarly, the result of in-vitro study of (Khodadadi et al. 2011) revealed that therapeutic index can be used as an index for assessment of safety profile and showed that, among the tested agents; the hydro-alcoholic extract of *Matricaria aurea* was a safer inhibitor of MMP-2 and MMP-9 than vitamin E and *Glycyrrhiza glabra.* The anti-parasitic efficacy of the solvent extract depends on intrinsic factors such as polarity of the solvent, nature of bioactive principles etc., and extrinsic factors *i.e.* method of extraction and storage and collection conditions of the extracts. In the present study, the solvent extract based on toxicological and efficacy parameters showed a distinct correlation and association between and among the group of solvent extract as depicted in 3. From the thorough study of Fig. 3, it can be congruent that LC_{50} and TI have positive correlation while AE and RPS has a positive correlation that validates the efficacy of the solvent extract of *T. arjuna* against *A. bengalensis*. Also, the association of these factors with solvent extract indicates the factor specific activity or association of the solvent extracts that has reflected in all treatment groups. In the present experiment the five best effective solvent extracts including one TAAF, TAAB (polar aprotic), TAMB, TAEB and TAMF showed the varied efficacy against *A. bengalensis* might be due to twofold reasons, firstly, capacity of a particular solvent to dissolve the bioactive principles in it and interactions with other bioactive constituent while dissolved in mother solvents or other neutral solvent system such DMSO, secondly, due to toxicity of the solvent systems but that can be negated by the fact that the LC_{50} value of a particular solvent extracts was much higher than the in-vitro and in-vivo experiments which clarified that the activity is mainly due to bioactive phytochemicals in it. Similar, results were also reported by many researchers (Gurib-Fakim 2006; Boursier et al. 2011; Shah et al. 2017; Kumari et al. 2019) who have claimed that the anti-parasitic efficacy of the extracts of Neam was due to presence of bioactive principles.

Further bibliographic study showed the research findings performed on various species of *Argulus*. For instance, recently, (Kumari et al. 2019) revealed that aqueous extract caused 100% mortality of adult *A. japonicus* with an acceptable level of the therapeutic index of 1.796 at 72 h under in vivo condition. (Kumar et al. 2012a, b) reported 100% mortality of *Argulus* at 20 mg/ L of azadirachtin solution under *in vivo* condition for 48 hours. (Mousa et al. 2008) and Mamadou et al. (2013) reported the 96 h LC_{50} of neem leaf extract (1.8 g/ L) and 36 hours LC_{50} (1271.22 mg/ L), respectively for juvenile *Oreochromis niloticus* and *Clarius gariepinus*. In contrast to these studies in the present experiment, the most effective solvent extracts; TAEB (*T. arjuna* ethanolic bark extract) showed 100 % mortality within 2 hours, 24 hours and 60 hours under in-vitro conditions (bath and immersion treatments), respectively. In contrast, the study of (Kumari et al. 2019), the ethanolic bark extract was found to be safe for the host. Similarly, TI was also lower 1.2, 1.1 and 1.6, in in-vitro, bath and immersion treatments, respectively, for TAEB than that the

previous studies. Noticeably, the marked variations in these factors the in present study, from the previous studies, might be attributable to the difference in the purity of the extract, nature of solvent extract, presence of bioactive principles and their effects, the difference in species of parasite and experimental condition.

Conclusions

Present study is the first of its kind to evaluate the anti-parasitic effects of five solvent extracts of *T. arjuna* against *A. bengalensis*. Out of five solvents, three TAEB, TAMB and TAAF were found to be potential solvent extract against *A. bengalensis* having TI values as; TAEB 1.1 (24 hour), 1.6 (60 hour) in bath and immersion treatments; TAAF 2.1 (24 hour), 1.6 (60 hour) under bath and immersion, respectively. Under bath treatment, TAEB could achieve 100 % anti-parasitic efficacy at 24 hours with 50mg/L concentration whereas in the case of immersion it was 60 hours at 40 mg/L concentration. Also, obtained ≥ 90% RPS in Bath treatment when parasitic challenged *Labeo rohita* exposed to the TAEB, TAMB and TAAF solvent extracts while in the case of immersion it was recorded ≤ 90% at 50 mg/L concentration. The TI values indicated that all effective solvent extracts found to be safe to the host and potential source for designing the bio-pesticide against fish ectoparasites including *A. bengalensis*. In addition, the solvent extracts were found as potential agents against moderately intensified (25-30 *A. bengalensis*/ fish) argulosis in *Labeo rohita* in all type of treatments. Although, the present study established a benchmark of five effective solvent extract of *T. arjuna*, however, prudently, more detailed studies on the active constituents of this ethnomedicinal medicinal plant need to be undertaken to utilize it in clinical practices.

Abbreviations RPS: Relative percentage survival; TI: Therapeutic index; LC50: Mean lethal concentration: EC-50: Mean effective concentration; TAAB: *Terminalia arjuna* acetone bark extract; TAAF: *Terminalia arjuna* acetone fruit extract; TAEB: *Terminalia arjuna* ethanol bark extract; TAMB: *Terminalia arjuna* methanol bark extract; TAAF: *Terminalia arjuna* fruit extract; AE: Anti-parasitic efficacy; DO: Dissolved oxygen: suffix IM: Immersion treatment: Suffix BA: Baath treatment; Suffix IN: In-vitro condition; 10, 20, 30, 40 and 50: stands for concentration in ppm

Ethical statement The present study follows the international, national, and/or institutional guidelines for humane animal treatment and complies with relevant legislation. The researcher has taken ethical approval from the institute and work was in compliance to guidelines mandatory for conducting the research.

Conflict of interest The authors declare that they have no conflict of interest.

Author Contribution Conceptualization: B.K.Das;Methodology: B.K.Das, N. P. Sahu and P.P. Srivastava; Softwares: D. K. Meena, A. K. Sahoo; Validation, D. K. Meena and H.S. Swain; Formal Analysis: D. K. Meena and S. Borah; Investigation:D. K. Meena and A. K. Sahoo; Resources: B. K. Das; Data Curation: D. K. Meena, B. K. Behera and S. Borah Writing – Original Draft Preparation: D. K. Meena, S. Borah and A. K. Sahoo; Writing – Review & Editing: D. K. Meena, B. K. Das and N. P. Sahu; Supervision: B. K. Das, N. P. Sahu and P.P. Srivastava Project Administration: B. K. Das.

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