

## RESEARCH ARTICLE

# Prospects and perspectives of virtual *in-vitro* toxicity studies on herbal extracts of *Terminalia arjuna* with enhanced stratagem in *Artemia salina* model: A panacea to explicit the credence of solvent system in brine shrimp lethality bioassay

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## ABSTRACT

An *in-vitro* toxicity study was undertaken for the validation, laydown the standards, supplement some of the information on assessment and define the nature of toxicity of different solvent extracts of *Terminalia arjuna* considering *Artemia salina* model, as a case study. In the present study, experimental conditions such as yellow color light of 100 W at 5% salinity for 48 hours with pH 8.0-8.5 at 30 °C, picking up of nauplii with 3mL dropper cut at its tip, 24 hours incubation in same experimental conditions and visualization with 50X magnifying glass were modified unlike previous studies. Functional screening of solvent extracts with their mother solvents revealed that hex, Etac, Chlo, Acet, Etoh, and Meoh exhibited, the LC<sub>50</sub> values as 118.50, 101.75, 93.36, 278.32, 528.78 and 477.67 ppm, respectively, designating them as; medium, medium, high, medium, low, medium toxic, while in their solvent extract forms the toxicity nature gets changed indicating the effectiveness of the extracts. Study, also defines the toxicity level for universal solvents such as DMSO and Dw as to be non-toxic as per the Meyers toxicity index and Clarkson's toxicity criterion. Among solvent extracts of *T. arjuna*, all were found to be toxic as per Meyers toxicity index. However, with reference to Clarkson's toxicity criterion, solvent extracts comprehended extended toxicity classes as low, medium and high toxic. The PCA 1 and 2 showed, 69.46% and 19.74% variations indicating strong correlation between the parameters. The results confirmed that LC<sub>50</sub> of any solvent extract could be treated as relative LC<sub>50</sub>, which is the fraction of mother solvent and could be treated as absolute LC<sub>50</sub>, which is the actual potential of the solvent extracts that might be positive for less effective and negative for highly effective solvent extracts. For smoothening of experimental results, negative sign was ignored. Relative LC<sub>50</sub>, relative LC<sub>50</sub>%, absolute LC<sub>50</sub>, and absolute LC<sub>50</sub>% could find liner relation with their % fractions and inverse relation with their counterparts. Thus present study advocates the inclusion of relative LC<sub>50</sub>, relative LC<sub>50</sub>%, absolute LC<sub>50</sub>, absolute LC<sub>50</sub>%, considering 95% upper and lower fiducial class intervals and TI values while fixing the nature of toxicity and designating the safety aspect to the host. Also, the study recommends for the collective effort to make BSLA as an internationally accepted and robust standard by revisiting and supplementing the existing toxicity criterion for the BSLA.

**Keywords:** BSLA; DMSO and *T. arjuna* solvents extracts; Relative and absolute LC<sub>50</sub>; Toxicity index and criterion; TI

## INTRODUCTION

Medicinal plants are considered the origin of the many therapeutic agents and novel drugs. In developing countries the medicinal plants are serving primary source of their health care. As per WHO (2007), major world populace (70-80%) are depending on plant based unconventional medicament as a primary treatment for the illness (Countries like China and India are serving as knowledge partner

globally in disseminating the ethno-medicinal data base on the medicinal plants. Although, medicinal plants are known to exhibit the excellent medical properties, some of them are possessing toxicants characteristics as well. India is considered as varietal emporium and having the richest diversity of medicinal plants. The *Terminalia arjuna* an important medicinal plant distributed around the world, has proved its strong foothold in terms of antimicrobial, anti-inflammatory, anti-oxidant, anti-diuretic and allergic and

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excellent homemade remedy for chronic cardiac ailments since ancient Ayurveda time. Despite of multifarious health benefits of *T. arjuna* it has not been explored scientifically for its toxicological properties and safe level of the solvent extracts for designing the novel drugs except some remote study on this aspect *i.e.* Suely et al. (2015) has reported that 80% ethanolic bark extract of *T. arjuna* was found to possess piscicidal effects on *Clarius batrachus* fish but has not confirmed with brine shrimp lethality test. Similarly, Bhatt et al. (2016) has screened only two solvent extracts (ethanolic and aqueous) and revealed the  $LC_{50}$  % as 20 and 10  $\mu\text{g/mL}$  for ethanolic and aqueous hot extracts, respectively, at 1000  $\mu\text{g/mL}$ . Toxicity of the plants extract are credence to the bioactive components. The toxicity of plants extracts has been assessed since long time by conventional approaches such as in-vivo and in-vitro toxicity studies taking other animals as model, and thereafter *Artemia salina* was mostly (90 %) used as model animal out of other species of the *Artemia* genus (Campbell et al., 1994). Owing to animal ethical concern in toxicity assessment experiment, there is need for alternate animal models. For evaluating the efficacy of the brine shrimp lethality assay (BSLA), a comparative study between the  $LC_{50}$  data of *Artemia salina* and acute toxicity in rats and mice was performed (Parra et al., 2001; Sharma et al., 2013; Naidu et al., 2014). Since, its inception, BSLA was adopted one amongst the user friendly strategy due to its beneficial aspects *i.e.* wider adaptability starting from herbal material (Meyer et al., 1982; McLaughlin et al., 1998a; Moshi et al., 2010; Ogugu et al., 2012; Gadir, 2012; Solanki and Selvanayagam, 2013; Sharma et al., 2013), toxicity of heavy metals (Sleet and Brendel, 1985; Martínez et al., 1999), to all the way for nano-particles (Maurer-Jones et al., 2013), heavy metals (Sleet and Brendel, 1985; Martínez et al., 1999) and metal ions (Kokkali et al., 2011, materials for dental applications (Pelka et al., 2000), and toxicity assessment of marine algae and cyanobacteria before bio-prospecting (Jaki et al., 1999; Mayorga et al., 2010; Carballo et al., 2002); toxicity of environmental media such as wastewater (Manfra et al., 2011), seawater (Manfra et al., 2010) and marine discharges (Nunes et al., 2006). Further, this is a simple, user friendly, economical, rapid, low requirement to startup (Hamidi et al., 2014), and bibliographic studies showed its competency and reliability in establishing correlation between the  $LC_{50}$  values ascertained in BSLA deploying *brine shrimp* and the outcomes from the mice model, adopting short-term toxicity assessment through oral mode of treatment (Parra et al., 2001; Arslanyolu and Erdemgil, 2006) bioassay as compare to their chemical and biological (MTT cell assay, zebra fish model) counter parts. Also, bio-markers and teratogenic substances, due to lower sensitivity are assumed to impose less impact in acute toxicity. (Yu and Lu, 2018). In addition, higher sensitivity

towards contaminant and stressful condition standout *Artemia salina* model as one of versatile one among other invertebrates (Van and Persoone, 1993). US Environmental Protection Agency (US EPA 1983) has documented by the *Artemia salina* ideal invertebrate for toxicological studies and and production checking. In spite of robustness of BSLA, some of the factors found to exhibit pragmatic role in deciding the efficacy and reliability of this assay. For instances, i), the mother solvents in which the test extract are prepared, play an orchestral dynamic in terms of toxicity of the herbal products. Some of the most commonly used organic solvent and detergents are reported to encompass high cyto-toxicity in vivo (Wu, 2014). Past studies showed DMSO, Tween-20, Nikkol (Wu, 2014) as solvents of choice and revealed maximum toxicity in as Tween-20, and apparently, DMSO and Nikkol designated as safe solvents but none of the study has conducted to evaluate the limit of toxicants and congruence of the solvents with combined toxicity of herbal products. ii) proper experimental design for drug delivery so that the interferences by these synthetic compounds can be abducted. Particularly, negative control group should be taken in consideration keeping that negative control can possess lesser amount of mother solvents. For real time value of toxicity, the value of solvents are to be deducted from the test samples. iii), the stage of *Artemia* to be used, as after hatching of *Artemia* cysts, past works showed a random selection of the stage to be used that inculcate the false or pseudo results. But references suggest that instar II-III could exhibit maximum sensitivity towards teratogenic substances (Vanhaecke et al., (1981) just after it exhausts its yolk sac. iv), the hatching and experimental conditions like pH, salinity, light, aeration, color and duration. v), selection of the appropriate and potential solvent of choice because the efficacy of herbal material greatly depends on the credence of solvent systems attributable to the bioactive principles. vi) optimization of various criterion of toxicity assessment such as behavioral studies and hatching ability but these can also give false results because of the not uniformity of uptake dose of toxicant by the nauplii incubated, and inference due to other factor which can also cause the erratic behavioral changes and swimming pattern during course of toxicity studies. Endpoints, observation by changes in behavioral activities such as erratic or interrupted swimming could have a prospective application in the establishment of electronic based technology. Image capturing and recording can be used for studying behavioral changes in thye organisms. *Artemia* spp. have been used since long time for landscaping of toxicants used in toxicological experiments (Dvorak et al., 2012), therefore, due to lack of stringent and globally accepted protocol, brine shrimp species could be utilized for assessment of the toxicity. In this back drop, an experiment was executed to stream line BSLA

for maximizing its sensitivity for carry out the plethora of toxicity studies taking solvent extracts of *T. arjuna* as a case study with consideration of its ethno-medicinal importance. Thus, the aim of above investigation is to validate earlier standards and laydown some principles that might be beneficial for toxicity and fortify some newer outcomes of the studies for making the BSLA more stringent and reliable toxicity assessment method.

## MATERIALS AND METHODS

### Preparation of artificial sea water and hatching of the *Artemia salina*

As the most important aspect for hatching of *Artemia* cysts is the salt water. For preparing artificial sea water, initially, 150 g of 200 g (5 %) common salt was added to the 4 L distilled and rest of 50 g was added next day water and mixed and filtered through fine mesh net of muslin cloth to removed extraneous material. To this 1 g *Artemia* cyst was added into two 5L capacity of cylindrical glass jar containing 4 L of water kept for obtaining the desired size and density (Fig. 1).

In these steps the inclusion of total quantity of salt required in two installments facilitates the size homogeneity of nauplii of *Artemia salina* which is very much important from the success of the experiment point of view. After providing, the 100 W capacity bulbs flashing yellowish light for enhancing the hatching success and proper aeration, the mouth of the jar was tightened with small mesh size net and kept the jars under observation. After 36 hours the active hatching started and up to 48 hours the hatching was observed.

### Preparation of the extracts

In the present study, the serial extraction was performed using same dry powder with different solvent based on their polarity. The extracts were prepared both in their mother solvents and DMSO at the stock concentration of 10, 20, 30, 40 and 50 ppm. The solvent extracts were



**Fig 1. Set-up of *Artemia salina* hatching unit.** The intervention includes in-terms of coloration, light flux, salinity, pH and temperature for ascertaining hatching, activity of 48 hours old Instar II nauplii of *A. salina* with maintaining size homogeneity. The experimental conditions were as follows: yellow color light of 100 v at 5% salinity for 48 hours with pH 8.0-8.5 at 30 °C, during hatching.

kept in amber glass sample container of 10 mL capacity to protect the bioactive constituents from oxidation and other chemical reactions.

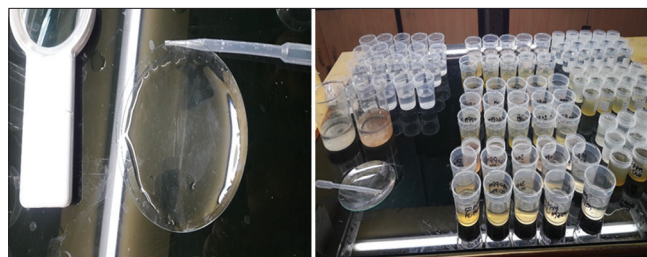
### Experimental set up and standardization of the experimental conditions

First of all, the mother solvents i.e. hexane, ethyl acetate, chloroform, acetone, ethanol, methanol, distilled water and reference solvent medium DMSO were taken for fixing the LC<sub>50</sub> values. Briefly, in a 50 mL capacity sample container (Tarson) 30 nauplii of *Artemia salina* container were added and 100-500 µL respective mother solvents were also added and incubated for 24 hours under 100 W yellow light and thereafter mortality was observed by taking no motion nauplii as dead and it was confirmed by visualizing under 50 X magnifying glass (Fig. 2).

The mortality was noted in all treatment groups. This is most important steps as lesson learnt from the previous studies and by trial and error method. The nauplii of *Artemia salina* of more than 48 hours old after achieving the instar II stage were taken into a 500 mL capacity beaker and thereafter with the help of 2 mL dropper which edge was cut with scissor to make it more convenient to pick the nauplii of *Artemia salina* in desired number in one go. For study purpose, the 4 L salt was also prepared in same manner as prepared for hatching the nauplii except whole calculated amount of salt was poured at time to make it 5% solution. For screening the *T. arjuna* solvent extracts, the conditions were followed as mentioned above and after putting nauplii into the 50 mL salt water, from the stock vials 500 µL was added (following fixed volume percentage method) to each container. The experiment was repeated thrice for confirmation with two elution mediums (mother solvent and DMSO) as mentioned and numbers of dead nauplii were noted against the treatment groups.

### Estimation of LC<sub>50</sub>, relative LC<sub>50</sub>%, absolute LC<sub>50</sub>, absolute LC<sub>50</sub>% and fixing the toxicity level

The LC<sub>50</sub> was estimated using Graph pad prism v.8.1.3 with excel sheet and percentage relative LC<sub>50</sub> and percentage



**Fig 2. Showing experimental set up and accessories.** The *in-vivo* study was conducted in laboratory for incubation in 50mL capacity sample containers and the nauplii picked up with the help of 3 mL dropper that cuts at the tip and 50 X magnifying glass was used to visualized the dead and alive nauplii upon 24 hours incubation.



absolute  $LC_{50}$  was calculated by the following formula considering the combined toxicity and absolute toxicity for assessing the actual toxicity of the particular solvent extract.

$$\text{Relative } LC_{50} (\%) = \frac{\text{Combined } LC_{50}}{LC_{50} \text{ of a solvent or solvent extract}} \times 100$$

$$\text{Absolute } LC_{50} (\%) = \text{Mother solvent } LC_{50} - \text{Relative } LC_{50}$$

Based on the  $LC_{50}$  values, the toxicity nature was fixed referring standard toxicity indices given by Meyer's and Clarkson's. As per the Meyer's toxicity classes extracts with  $LC_{50} < 1000 \mu\text{g/mL}$  (toxic), and  $LC_{50} > 1000 \mu\text{g/mL}$  (non-toxic) non-toxic (Meyer et al., 1982).

Clarkson's classified as follows:  $LC_{50}$  above  $1000 \mu\text{g/mL}$  (toxic),  $LC_{50}$  500 -  $1000 \mu\text{g/mL}$  (low toxic),  $LC_{50}$  100 -  $500 \mu\text{g/mL}$  (medium toxic), and  $LC_{50}$  0 -  $100 \mu\text{g/mL}$  (highly toxic) (Clarkson et al., 2004).

## RESULTS

### Experimental set up and standardization of the experimental conditions

The improved experimental conditions such as salinity, light duration, color and intensity have better toxicity assessment. The 5 % common salt water showed size uniformity and validate the duration for obtaining the instar II stage after 48 hours. The yellow light source of 100 W induces the activity of nauplii that reduces the chances of experimental error because the dose of solvent extract would pursue better to the active nauplii that can be easily assessed for

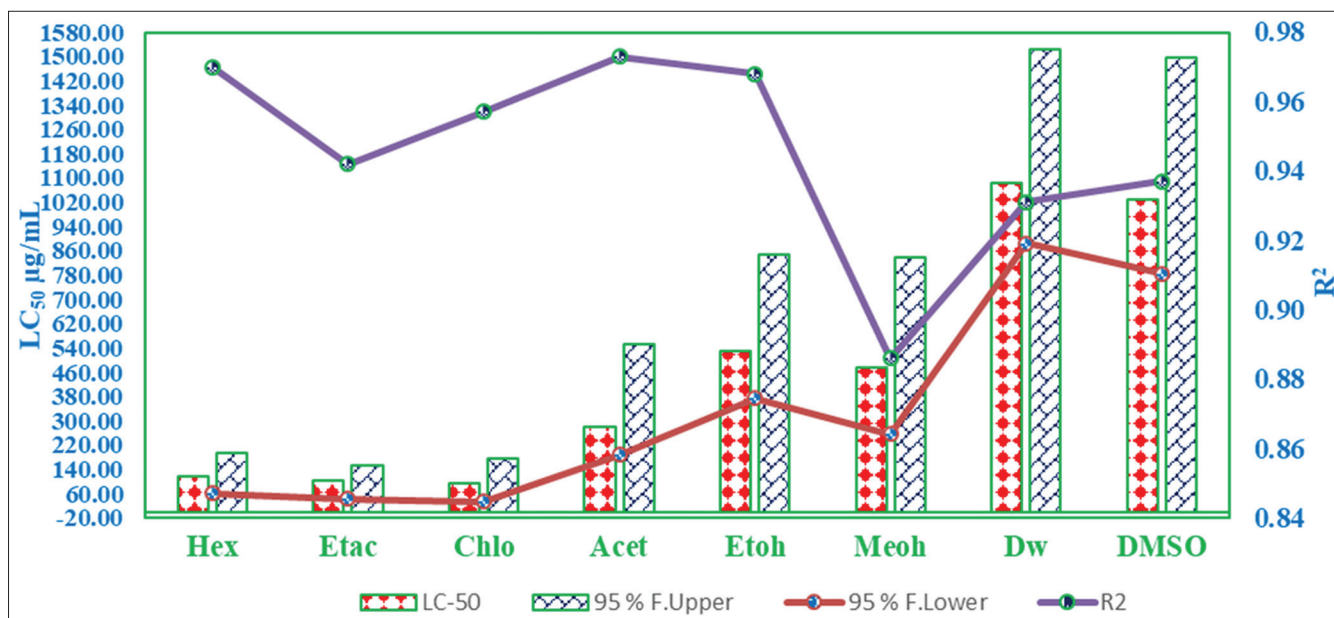
the mortality. Similarly, pH and temperature were kept  $7^\circ\text{C}$  and  $30^\circ\text{C}$  for entire experimental period starting from cysts hatching to end of the toxicity assessment would result in better survival and robustness to the nauplii.

### Estimation of $LC_{50}$ , absolute $LC_{50}$ , absolute $LC_{50} \%$ , relative $LC_{50} \%$ , and fixing the toxicity level

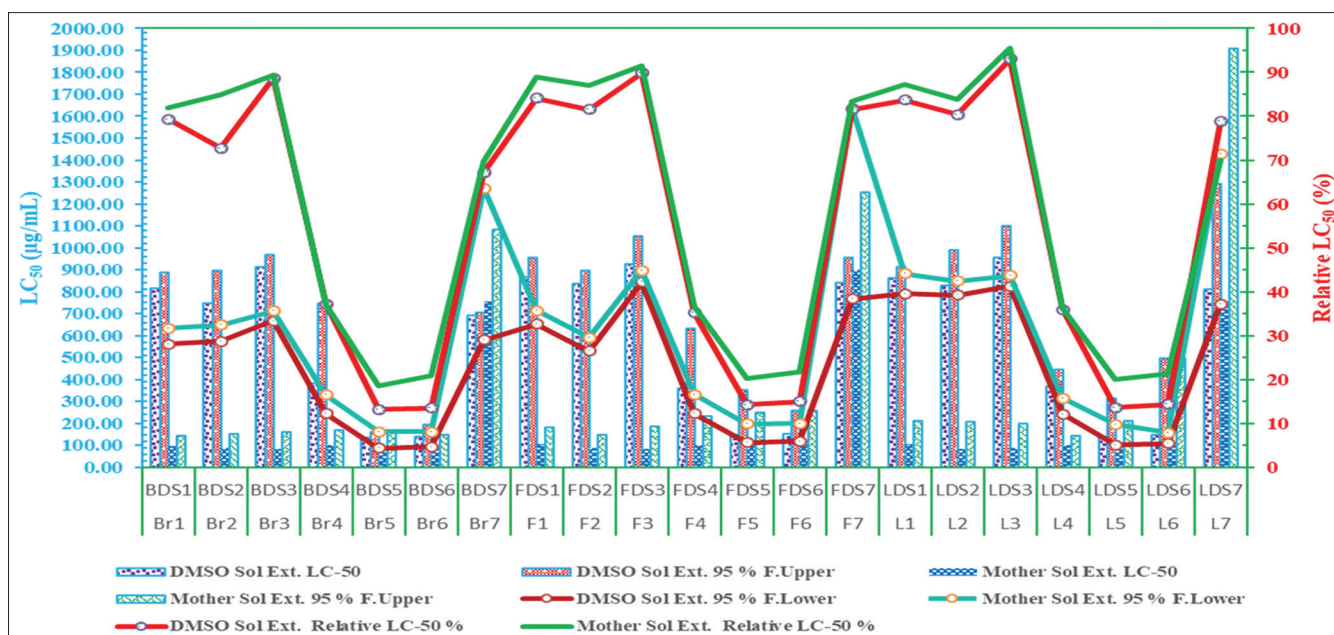
Fig. 3, briefly illustrates, the comparative toxicological parameters of mother solvent extracts and DMSO solvent extracts. The mother solvents and DMSO are being plotted on X-axis while primary Y-1 axis and primary Y-2 axis showing  $LC_{50}$  value and  $R^2$  values for the respective solvent systems.

The  $LC_{50}$  values for mother solvents and DMSO in ascending order can be presented as Dw ( $1081.30 \mu\text{g/mL}$ ) > DMSO ( $1029.00 \mu\text{g/mL}$ ) > EtOH ( $528.78 \mu\text{g/mL}$ ) > MeOH ( $477.67 \mu\text{g/mL}$ ) > Acet ( $278.32 \mu\text{g/mL}$ ) > Hex ( $118.51 \mu\text{g/mL}$ ) > Etac ( $101.75 \mu\text{g/mL}$ ) and Chlo ( $93.36 \mu\text{g/mL}$ ). The 95 % upper fiducial class interval were as follows; Dw ( $1524.11 \mu\text{g/mL}$ ), DMSO ( $1495.78 \mu\text{g/mL}$ ), EtOH ( $846.51 \mu\text{g/mL}$ ), MeOH ( $840.40 \mu\text{g/mL}$ ) and Acet ( $552.00 \mu\text{g/mL}$ ). Similarly, the 95 % upper fiducial class interval showed same trend for lower 95% lower fiducial limit. The  $R^2$  value of solvents correlated as Hex (0.97)  $\geq$  Acet (0.97)  $\geq$  EtOH (0.97) > Chlo (0.96) > Etac (0.94)  $\geq$  DMSO (0.94) > Dw (0.93) > MeOH (0.93).

In Fig. 4, the primary X-axis and secondary X-axis showing, the DMSO solvent extracts and mother solvent extracts, respectively. Similarly, primary and secondary Y- axis, showing  $LC_{50}$  ( $\mu\text{g/mL}$ ) and relative  $LC_{50} \%$ , respectively. The



**Fig 3. Showing comparative toxicological parameters of mother solvents and DMSO solvent.** The mother solvents and DMSO are being plotted on X-axis while primary Y-1 axis and primary Y-2 axis showing  $LC_{50}$  value and  $R^2$  values for the respective solvent systems.



**Fig 4.** Showing comparative toxicological parameters of mother solvent extracts and DMSO solvent extracts. DMSO solvent extracts and mother solvent extracts were plotted on primary X-axis and secondary X-axis respectively. Similarly,  $LC_{50}$  ( $\mu\text{g/mL}$ ) and relative  $LC_{50}\%$ , was depicted on primary and secondary Y- axis, respectively.

maximum  $LC_{50}$  among DMSO solvent extracts showed by LDS3 (957.15  $\mu\text{g/mL}$ ) followed by FDS3 (924.07  $\mu\text{g/mL}$ ), BDS3 (912.32  $\mu\text{g/mL}$ ) whereas lowest  $LC_{50}$  showed by BDS5 (135.99  $\mu\text{g/mL}$ ) followed by BDS6 (139.01  $\mu\text{g/mL}$ ), LDS5 (139.94  $\mu\text{g/mL}$ ).

The relative  $LC_{50}\%$  was recorded maximum in L3 (95.51  $\mu\text{g/mL}$ ) and LDS3 (93.01  $\mu\text{g/mL}$ ) followed by F3 (91.38  $\mu\text{g/mL}$ ) and FDS3 (89.38  $\mu\text{g/mL}$ ). Among mother solvent extracts, the lowest  $LC_{50}$  was recorded for Br3 (83.45  $\mu\text{g/mL}$ ) followed by L2 (85.35  $\mu\text{g/mL}$ ), Br2 (86.46  $\mu\text{g/mL}$ ). The maximum 95% fiducial upper class interval value of  $LC_{50}$  was observed for LDS7 (1290.23  $\mu\text{g/mL}$  and L7 (1907.94  $\mu\text{g/mL}$ ) as DMSO solvent extract and mother solvent extract, respectively. The lowest fiducial class interval was reported for Br5 (75.03  $\mu\text{g/mL}$ ) and BDS5 (75.03  $\mu\text{g/mL}$ ).

The reference  $LC_{50}$  values for DMSO solvent extracts were recorded as to be 1029.08  $\mu\text{g/mL}$  and for mother solvent extracts such as Hex, Etac, Chlo, Acet, Etoh, Meoh and Dw, it was, 118.51, 101.75, 93.36, 278.32, 528.78, 1081.30  $\mu\text{g/mL}$ , respectively. The maximum relative  $LC_{50}$  for DMSO solvent extracts was found to be as 957.17 for LDS3, followed by FDS3 (924.07) and BDS3 (912.32) and lowest in case of BDS5 (135.99) followed by BDS6 (139.01) and LDS5 (139.94). Among mother solvent extracts, the maximum value for relative  $LC_{50}$  was reported for F7 (900.94) followed by L7 (757.99) and Br7 (755.60) and lowest were noted for Br3 (83.45) followed by F3 (85.31) and L3 (89.16). The absolute  $LC_{50}$  fraction value is considered

as the potential of the solvent extracts. The maximum  $LC_{50}$  fraction among DMSO solvent extracts, was found to be in BDS5 (893.09) followed by BDS6 (890.07), LDS5 (889.14), FDS5 (882.09) and FDS6 (874.71). The % of relative and absolute  $LC_{50}$  value also followed the same trend, and maximum % relative  $LC_{50}$  was recorded as 95.51 for L3 which is followed by F3 (91.38) and Br3 (89.39) and lowest was 18.66 for Br5 followed by L5 (20.00) and F5 (20.31) among mother solvent extracts. While % absolute  $LC_{50}$  value was maximum in Br5 (81.34) followed by L5 (80.00), F5 (79.69), Br6 (79.03), L6 (78.61) and F6 (78.15) and lowest was 4.49 for L3 followed by F3 (8.62), Br3 (10.61), F1 (11.17) and L1 (12.73) among mother solvent extracts. Among, DMSO solvent extracts maximum value for % relative  $LC_{50}$  value was recorded in BDS5 (86.79) followed by BDS6 (86.49), LDS5 (86.40) and FDS5 (85.72) whereas lowest was found to be as 4.49 for LDS3 followed by FDS3 (10.20) and BDS3 (10.61).

Table 1. depicting the toxicity levels, nature of mother solvents and their solvent extracts and DMSO and its solvenet extracts. As per Meyer's toxicity index and Clarkson's toxicity criterion, only DMSO and Dw having  $LC_{50}$  value as 1029.08  $\mu\text{g/mL}$  and 1081.30  $\mu\text{g/mL}$ , respectively, were found to be non-toxic. As per the Meyers toxicity index, rest of the solvent extract were found to be toxic whereas in Clarkson's toxicity criterion, the toxic compounds are again divided into three categories. i.e. low toxic, medium toxic and highly toxic. According to this criterion, DMSO solvent extract fall under low or medium toxic categories while mother solvent extracts occupied each category

**Table 1: Showing the toxicity nature and other toxicological parameters of Mother solvents and DMSO solvent and their solvent extracts**

Extract	LC <sub>50</sub> (µg/mL)	Absolute LC <sub>50</sub> (µg/mL)	95 % Fiducial CI lower	95 % Fiducial CI upper	R <sup>2</sup>	Nature of toxicity	
						Meyer's toxicity index	Clarkson's toxicity criterion
DMSO	1029.08	NA	436.018	1595.78	0.93	non-toxic	*non- toxic
BDS1	815.91	213.17	560.91	888.91	0.99	toxic	**low toxic
BDS2	748.34	280.74	574.46	894.20	0.98	toxic	**low toxic
BDS3	912.32	116.75	667.71	968.94	0.95	toxic	**low toxic
BDS4	383.22	645.85	245.73	749.60	0.93	toxic	**low toxic
BDS5	135.98	893.09	87.79	159.38	0.90	toxic	***medium toxic
BDS6	139.01	890.07	94.33	195.77	0.94	toxic	***medium toxic
BDS7	691.14	337.94	580.16	706.60	0.98	toxic	**low toxic
FDS1	864.94	164.14	653.15	955.73	0.96	toxic	**low toxic
FDS2	838.72	190.35	529.20	896.07	0.94	toxic	**low toxic
FDS3	924.07	105.01	843.98	1051.91	0.93	toxic	**low toxic
FDS4	362.63	666.45	245.60	633.85	0.94	toxic	***medium toxic
FDS5	146.98	882.09	113.98	351.91	0.93	toxic	***medium toxic
FDS6	154.37	874.71	120.28	260.10	0.99	toxic	***medium toxic
FDS7	839.099	189.97	768.63	954.39	0.95	toxic	**low toxic
LDS1	860.79	168.29	790.85	911.82	0.98	toxic	**low toxic
LDS2	826.76	202.31	783.93	988.78	0.97	toxic	**low toxic
LDS3	957.15	71.92	825.36	1099.08	0.93	toxic	**low toxic
LDS4	369.78	659.29	242.17	443.84	0.99	toxic	***medium toxic
LDS5	139.94	889.13	103.02	313.41	0.95	toxic	***medium toxic
LDS6	147.95	881.12	109.10	496.79	0.96	toxic	***medium toxic
LDS7	810.22	218.85	745.11	1290.23	0.98	toxic	**low toxic
Hex	118.51	NA	60.75	193.82	0.97	toxic	***medium toxic
Etac	101.74	NA	43.74	152.78	0.94	toxic	***medium toxic
Chlo	93.36	NA	35.26	274.54	0.96	toxic	****highly toxic
Acet	278.32	NA	88.24	551.99	0.97	toxic	***medium toxic
Etoh	528.78	NA	74.12	846.51	0.97	toxic	**low toxic
Meoh	477.67	NA	55.77	840.41	0.89	toxic	***medium toxic
Dw	1081.29	NA	585.21	1524.11	0.93	non-toxic	*non-toxic
Br1	97.09	21.41	72.13	145.53	0.98	toxic	****highly toxic
Br2	86.45	15.29	73.74	150.79	0.97	toxic	****highly toxic
Br3	83.45	9.91	45.00	161.97	0.88	toxic	****highly toxic
Br4	102.87	175.47	83.52	169.30	0.95	toxic	***medium toxic
Br5	98.65	430.13	75.03	166.61	0.93	toxic	****highly toxic
Br6	100.16	377.51	66.80	147.68	0.95	toxic	****highly toxic
Br7	755.59	325.70	690.33	1084.73	0.98	toxic	**low toxic
F1	105.26	13.24	61.13	181.27	0.96	toxic	***medium toxic
F2	88.45	13.28	61.69	147.36	0.91	toxic	****highly toxic
F3	85.31	8.051	54.20	186.44	0.94	toxic	****highly toxic
F4	102.14	176.19	85.60	233.85	0.93	toxic	***closed to highly toxic/medium toxic

(Contd...)

Table 1: (Continued)

Extract	LC <sub>50</sub> (µg/mL)	Absolute LC <sub>50</sub> (µg/mL)	95 % Fiducial CI lower	95 % Fiducial CI upper	R <sup>2</sup>	Nature of toxicity	
						Meyer's toxicity index	Clarkson's toxicity criterion
F5	107.35	421.37	83.98	251.91	0.93	toxic	***closed to highly toxic/medium toxic
F6	104.35	373.31	80.28	260.10	0.98	toxic	***closed to highly toxic/medium toxic
F7	900.93	180.36	868.63	1254.39	0.95	toxic	**low toxic
L1	103.42	15.08	90.85	211.82	0.98	toxic	***closed to highly toxic/medium toxic
L2	85.34	16.40	63.93	208.78	0.97	toxic	****highly toxic
L3	89.16	4.19	48.45	199.23	0.97	toxic	****highly toxic
L4	100.88	177.43	72.17	143.84	0.99	toxic	****highly toxic
L5	105.73	423.048	93.02	213.41	0.95	toxic	***closed to highly toxic/medium toxic
L6	102.19	375.48	49.10	496.79	0.96	toxic	***closed to highly toxic/medium toxic
L7	757.98	323.31	684.21	1907.94	0.83	toxic	low toxic

\*Indicate the lower value of absolute LC<sub>50</sub> as compared to relative LC<sub>50</sub>; \*\* indicate the higher value of absolute LC<sub>50</sub> as compared to relative LC<sub>50</sub>

i.e. Br1-Br3 (LC<sub>50</sub>: 97.09, 86.46, 83.45 µg/mL); Br5-Br6 (LC<sub>50</sub>: 98.65, 100.16 µg/mL); F1-F2 (LC<sub>50</sub>: 88.46, 85.31); L2-L4 (LC<sub>50</sub>: 85.35, 89.16, 100.88) as highly toxic, and medium toxic or closed to highly toxic were; Br4(102.87), F4-F6 (LC<sub>50</sub>: 102.13, 107.41, 104.36 µg/mL), L1:103.42, L5-L6 (LC<sub>50</sub>: 105.73, 102.19 µg/mL) whereas Br7, F7 and L7 were observed to be as low toxic with LC<sub>50</sub> value, 755.60, 900.94 and 757.99 µg/mL, respectively. Among mother solvents, Hex and Etac (closed to highly toxic), Acet and Meoh were reported to be as medium toxic with LC<sub>50</sub>: 118.51 µg/mL, 101.75 µg/mL, 278.32 µg/mL and 477.67 µg/mL, respectively while Chlo: LC<sub>50</sub>, 93.35 µg/mL was found to be highly toxic and Etoh as low toxic LC<sub>50</sub>, 528.78 µg/mL.

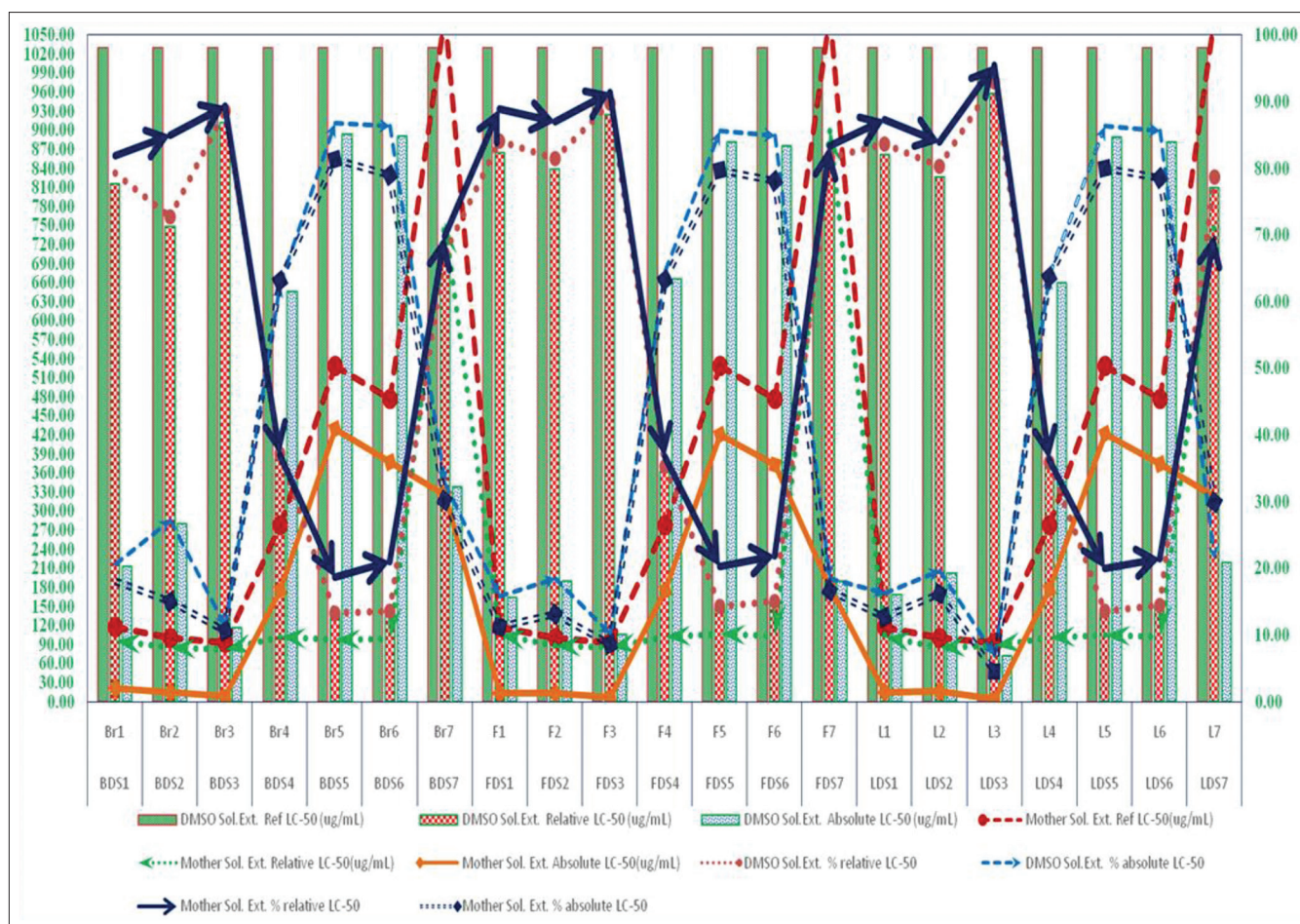
## DISCUSSION

### Prospects

The BSLA is considered a rapid result generating, reliable, economical and suitable test for toxicity assessment (Meyer et al., 1982). The procedure defines the LC<sub>50</sub> values in µg/mL of bioactive principles of test materials in the salt conditions. The credence of activities of bio-efficient principles and test substances are demonstrated as to be toxic to shrimps. The past research on the bioassay revealed that it exhibits strong correlation with cytotoxic activity against tumor proliferating tissues in human being, and has directed to the investigation of elite group of usual anti-carcinogenic agents concomitant with ethno-medicinal important plants (McLaughlin et al., 1998). Besides, the bio-efficacy of some herbal materials in BSLA, were reported to comprehend strong anti-oxidant potential, anticancer, antitumor, antiparasitic and antimicrobial potential

which might be archived to the occurrence of secondary metabolite such as phenolics, flavonoids, terpenoids etc (Lee, 1992; Ren et al., 2003; Tungmunthum et al., 2018; Aryal et al., 2019). Despite of great scope of BSLA in toxicity assessment, there is presently no internationally accepted standard. For this reason, calibrations with existing standards and continuous upgradation for standardization is highly recommended (Manfra et al., 2015). It has been reported that amongst the commonly used endpoints linking to short-term mortality, long-term mortality, acute mortality, hatchability of cysts and behavioral dynamics were calibrated based on the existing (Manfra et al., 2015), while long-term hatchability calibrated based on Italian standards. Present study highlighted the Toxicity level/nature of different solvent extract of the *T. arjuna* beneficial ethno-medicinal plant with modification in the existing BSLA. The study complied non-toxic nature of DMSO and Distilled water, however, DMSO was found to retain some toxic activity as compared to the distilled water which is supported with low LC<sub>50</sub> value for DMSO that also in accordance to the study of Worthley and Schott (1967) whose study implies that LC<sub>50</sub> increment was correspond to the increasing concentration and was found to be significant between 50% and 25% incorporation of DMSO. Present study pursuit better results of *Artemia salina* hatching including active movement and uniform size might be possibly due to alternation in salt dose while preparing artificial sea water and duration, color, temperature and capacity of light that was used and maintained as 100 W yellow light for 48 hours at 30 °C with salt water pH 8.0-8.5 that was modified from past studies (Vanhaecke et al., 1980; Guzzella 1997; Artookit 2014; Manfra et al., 2016). Libralato et al. (2016) reviewed the changes in previous studies based on trial and error methods and suggested short term (24 h) and long





**Fig 5.** Showing comparative combined toxicological parameters for mother solvent extracts and DMSO solvent extracts. The primary and secondary, X-axis reflecting solvent mother solvent extracts and DMSO solvent extracts, respectively. Primary Y-axis showing different toxicity classes where as secondary Y-axis toxicity parameters to be represented in %.

term (14 d) mortality as to be the prominent *Artemia* spp. protocols that might with stand to international standards. The deviation of the previous results from present study is also evidence that might be due to species specific nature, source of collection and treatment conditions. As far as the toxicity studies are concerned the *Artemia salina* used in the present study was found to achieve Instar stage II that recommended for toxicity studies as earlier stage are more resistant that may alter the actual results (Hamidi et al., 2014). In the present study total salt needed for preparing artificial sea water was provided in two installments of which approx. 75 % as primary and 25 % as secondary dose that were given next morning after setup of the hatching experiment. The possible reason behind the better result is the diffuse salt doses which could enhance. salt resistance to the species and thereafter provoked adaptive gene interactions which also providing strength and capacity to survive and actively move in the medium, and also provides filtered result of toxicity as the *Artemia salina* would be practically more harder than normal one After, hatching, the toxicity experiment was setup by picking up the nauplii of *Artemia*

*salina* with 3mL dropper that was cut at the tip to ensure hassle free required number of nauplii and put into 50 mL plastic container with optimized experimental conditions as mentioned above unlike to the previous studies who suggested to set up the toxicity testing either by pre-treating the Petri dish with light or short term light exposure for 1 hour and then incubation for 24 hours in dark (Artoxkit® 2014). This is in contrast to our study which used continuous light source that is supported by Sorgeloos, (1973) who noticed that light sensation was found to expedite hatching of brine shrimp cysts significantly and illustrated that embryological progression of hydrated embryos that are not ignited by light, can be interrupted till the light appear. As a thumb rule, hatching of cysts after light stimulus can make paramount differences for dark and light experimental conditions(Sorgeloos, 1973). The small deviation can make large differences while set up of experiments, for instances, picking the larvae with micropipette exert extraneous pressure while picking up the sensitive early stage of *Artemia* to reach instar II that is to be used for toxicity studies. The reason of suitable stage of *Artemia* has briefly illustrated



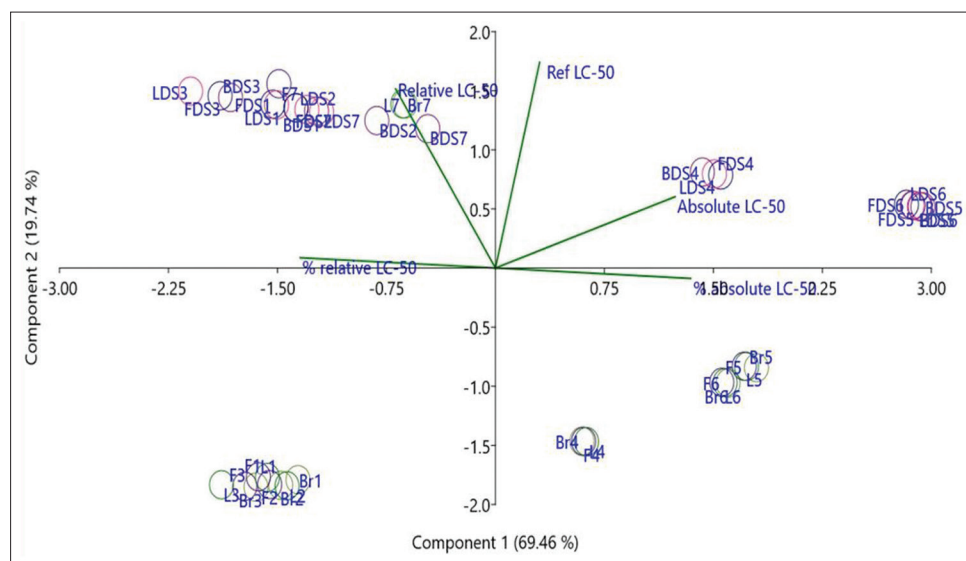
by many researchers (Sorgeloos et al., 1978; Vanhacck and Persoone, 1984; Sleet and Brendel, 1985; Kokkali et al., 2011). It has been demonstrated that I instar is the only stage which can efficiently use yolk as a primary source for food supply and found to exhibit more tolerance against chromic acid, as this stage could not develop efficient digestive tract essential for facilitating absorption of incoming materials from the elementary canal. The Table 2, is showing the comparative toxicological parameters of BSLA of *T. arjuna* solvent extracts. Most noticeably, in present study the author emphasized upon the finding that  $LC_{50}$  of the solvent extract are the relative  $LC_{50}$  correspond to the respective mother solvents and absolute value should be the actual value of the extract alone. As, it is known that negative control could be used for eliminating the other fraction contributing in the overall toxicity. The solvent system deployed to mix the evaporated plant materials is a relevant control for this purpose so actual value should be obtained when the  $LC_{50}$  of mother solvent to be deducted from relative  $LC_{50}$  of corresponding extract but present study indicates that mother solvent has more  $LC_{50}$  as compared to the solvent extract. If we take real value then absolute  $LC_{50}$  value of effective extract would be always in negative or if we take absolute value ignoring the sign then it would be higher than the relative  $LC_{50}$  Value of the solvent extracts. From the perusal of Fig. 4 and Table 2, it can be implied that relative  $LC_{50}$  and absolute  $LC_{50}$  has inverse relation that may likely due to the fact that relative  $LC_{50}$  value is toxicity contributed due to mother solvents and vice versa.

Fig. 6 showed that % relative  $LC_{50}$  values and % absolute value has negative correlation and similarly, relative  $LC_{50}$  value and Absolute  $LC_{50}$  values are negatively correlated, and reference  $LC_{50}$  that is the  $LC_{50}$  of

mother solvents, are highly positively correlated with the absolute  $LC_{50}$  values. Among solvent extracts, the BDS4, FDS4, LDS4 and BDS5 & 6; LDS5 & 6; FDS5 & 6 are highly correlated with reference  $LC_{50}$  values of solvent extracts.

Similarly, Fig. 7 showed the distribution pattern of solvent extracts and clearly depicting that effective extract exhibiting more absolute  $LC_{50}$  than the relative  $LC_{50}$  values. These results are in accordance to the Musa (2012) who has suggested that  $LC_{50}$  ( $240 \pm 3 \mu\text{g/mL}$ ) value of 80% acetone extract was found to be more effective as compared to n-butanol extract  $LC_{50}$  ( $437 \pm 8 \mu\text{g/mL}$ ). Similarly, the toxicity of ethanolic extract in other study also showed mild toxicity and considered as safe but in our study the ethanolic bark extract showed elevated toxicity might be due to presence of some of the active ingredients which is in conformity to the previous research (Tungmunthum et al., 2018; Aryal et al., 2019).

In the present study, the polar solvents such as ethanol, methanol and water are; low, medium and non-toxic while in solvent extracts forms their toxicity nature gets changed to closed to highly toxic and as per the toxicity criterion it falls as medium toxic or highly toxic which not an proof of being safe or unsafe as far as their applications in human and other vertebrates are concerned. For instances, the *T. arjuna* are being used as aqueous hot extract (decotion) and crude forms are reported to be very much effective against chronic ailments and disease without negatively affecting the physiology of an organism (Chaudhari and Mahajan, 2015).

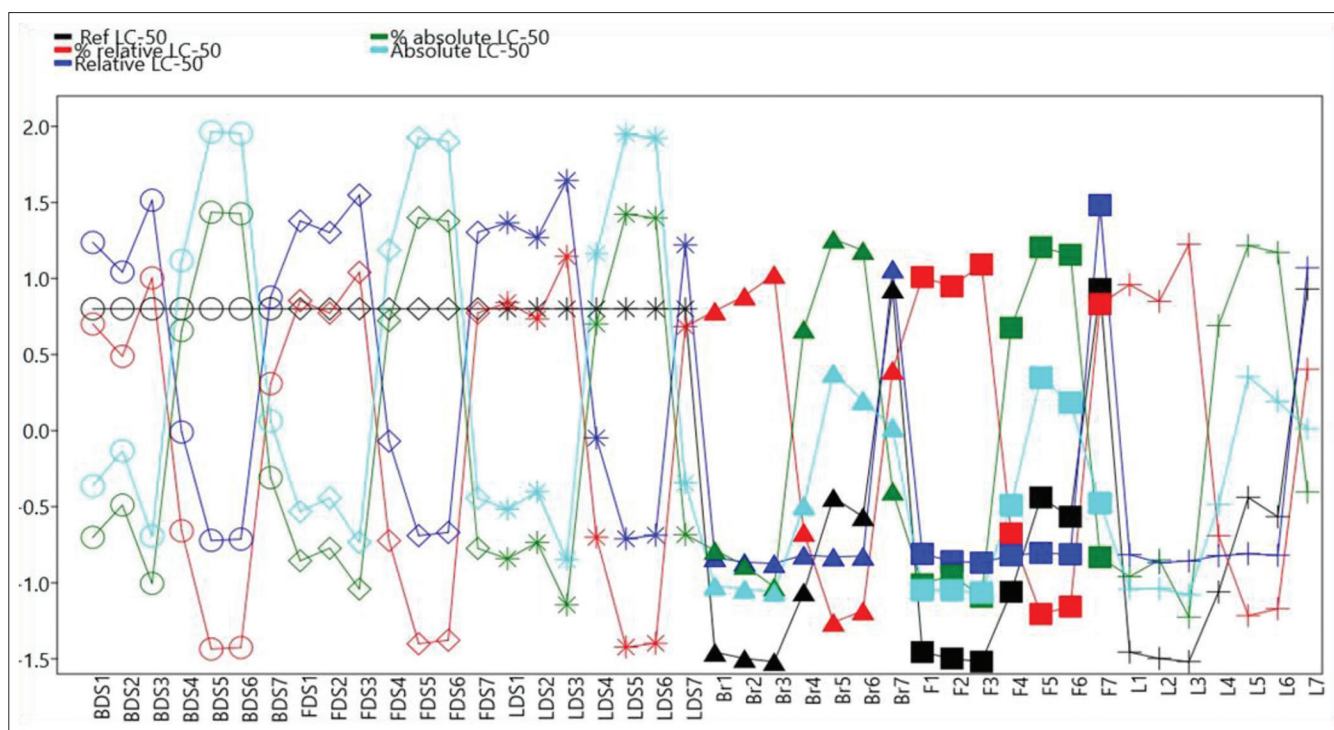


**Fig 6. PCA bi-plot of *T. arjuna* solvent extract with toxicological parameters.** Figure showing the correlation between solvent extracts and toxicological parameters. The PCA components explaining the variations among the parameters.

Table 2: Showing the combined data of BSLA of *T.arjuna* solvent extracts

	Ref LC <sub>50</sub>	% relative LC <sub>50</sub>	Relative LC <sub>50</sub>	% absolute LC <sub>50</sub>	Absolute LC <sub>50</sub>
BDS1	1029.07	79.28	815.90	20.72	*213.17
BDS2	1029.07	72.72	748.34	27.29	*280.74
BDS3	1029.07	88.65	912.32	11.35	*116.75
BDS4	1029.07	37.24	383.22	62.76	**645.85
BDS5	1029.07	13.21	135.99	86.78	**893.091
BDS6	1029.07	13.51	139.01	86.49	**890.06
BDS7	1029.07	67.16	691.14	32.84	*337.93
FDS1	1029.07	84.05	864.94	15.95	*164.13
FDS2	1029.07	81.50	838.73	18.45	*190.34
FDS3	1029.07	89.79	924.07	10.20	*105.01
FDS4	1029.07	35.24	362.63	64.76	**666.44
FDS5	1029.07	14.28	146.98	85.72	**882.09
FDS6	1029.07	15.01	154.37	84.99	**874.71
FDS7	1029.07	81.54	839.09	18.46	*189.97
LDS1	1029.07	83.65	860.78	16.35	*168.29
LDS2	1029.07	80.34	826.76	19.66	*202.31
LDS3	1029.07	93.01	957.15	6.98	*71.92
LDS4	1029.07	35.93	369.78	64.06	**659.29
LDS5	1029.07	13.59	139.94	86.40	**889.13
LDS6	1029.07	14.38	147.95	85.62	**881.12
LDS7	1029.07	78.73	810.22	21.26	*218.85
Br1	118.51	81.93	97.09	18.06	*21.41
Br2	101.75	84.97	86.45	15.02	*15.29
Br3	93.35	89.39	83.45	10.61	*9.91
Br4	278.32	36.96	102.87	63.039	*175.45
Br5	528.78	18.67	98.65	81.34	**430.12
Br6	477.67	20.97	100.16	79.03	**377.51
Br7	1081.29	69.89	755.59	30.12	*325.71
F1	118.51	88.83	105.26	11.17	*13.24
F2	101.75	86.94	88.46	13.06	*13.28
F3	93.3	91.37	85.31	8.62	*8.05
F4	278.32	36.69	102.13	63.31	**176.19
F5	528.78	20.31	107.41	79.68	**421.37
F6	477.67	21.85	104.36	78.15	*373.31
F7	1081.29	83.32	900.94	16.68	*180.36
L1	118.51	87.27	103.42	12.73	*15.08
L2	101.75	83.88	85.35	16.12	*16.40
L3	93.36	95.51	89.16	4.49	*4.19
L4	278.32	36.25	100.89	63.75	**177.43
L5	528.78	19.99	105.73	80.01	**423.04
L6	477.67	21.39	102.19	78.61	**375.48
L7	1081.29	70.09	757.99	29.90	*323.31

\*Indicate the lower value of absolute LC<sub>50</sub> as compared to relative LC<sub>50</sub>; \*\* indicate the higher value of absolute LC<sub>50</sub> as compared to relative LC<sub>50</sub>



**Fig 7. Distribution of toxicological parameters of *T. arjuna* solvent extracts by BSLA.** Figure showing the competence of the effective solvent extracts in terms of exhibiting more absolute  $LC_{50}$  than the relative  $LC_{50}$  values.

### Perspectives

1. A holistic approach is needed to streamline the unwind aspects of toxicity in context of classes of toxicity and to declare *Artemia* as globally accepted model in eco-toxicology involving institutional and regulatory mechanism enforced by regulatory and apex statutory body to define the toxicity level of unexplored aspects of safe and being toxic.
2. The toxicity studies should take lower and upper 95 % Fiducial Class interval into account while deciding the nature of the herbal extracts and corresponding  $LC_{50}$  value to be laid between and upper and lower limit.
3. The concept of relative and absolute  $LC_{50}$  need to be implemented so that the actual toxicity potential and strength of the herbal or solvent extracts can be plotted by eliminating the effects of negative control in toxicity assessment.
4. The study suggest that polar solvent extract of ethanol, methanol and distilled water found to be medium to low toxic, non-toxic which facilitates the possibility of their inclusion as mother solvents for extraction and as an alternate to DMSO for short term toxicity provided the relative and absolute  $LC_{50}$  are known for the solvent extracts.
5. The existing criterion and index of toxicity assessment need a through revision and modification in terms of fixing the toxicity of a particular extracts. For instances, in the present study, as per the Meyers toxicity index

all solvent extract are found to be toxic except distilled water and DMSO whereas in Clarkson's criterion, the solvent extracts found to be taking places in different classes *i.e.* non, low, medium, and highly toxic. Some of the solvents were exhibited very narrow margin to a particular level and the range of toxicity level is too wide to define it properly, therefore, there is need to expand and define more classes considering the ethno-medicinal uses of a particular herbal material.

6. The toxicity criterion needs to be supplemented by the therapeutic index (TI) and  $EC_{25} \% \leq LC_{50}$  value of solvent extracts while determine the toxicity nature of the extracts mere based on BSLA so that the declaration with regards to safety aspects and possibility of designing new drugs and medicine might be sound and reliable practice.

### CONCLUSION

The BSLA study of solvent extracts of *T. arjuna* validated the toxicity nature of the corresponding solvent extracts with  $LC_{50}$  of brine shrimp, however, to enhance the robustness of the method, there is need to revisit the existing toxicity criterion in terms of actual  $LC_{50}$  value and the strength of the solvent extract. More scientifically, the inclusion of two new terminologies, relative and absolute  $LC_{50}$  should be in place. In addition, the consideration of absolute  $LC_{50}$  if the value exceeds from relative  $LC_{50}$ ,



as potential of the solvent extract as being toxicant. The ethanolic and methanolic bark extracts could exhibit maximum absolute LC<sub>50</sub> % fractions out of total toxicity which showed their potential as safe therapeutic agents.

### Conflicts of interest

Authors declare no conflicts of interest and any financial competitiveness for the present work. The research is an original work neither published nor under consideration for publication anywhere.

### Contribution of the author

B. K. Das: Designed and monitored the research work; N. P. Sahu: Designed and checked draft manuscript; P.P. Srivastava: Designed the experiment; A. K. Sahoo: Manuscript preparation and data analysis; D. K. Meena: Research, draft manuscript preparation and statistical analysis; S. Borah: Data analysis; H.S. Swain: *Artemia* culture.

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