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REVIEW ARTICLE

Artemia species: An Important Tool to Screen General Toxicity Samples

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Abstract: Medicinal plants are a good source of novel therapeutic drugs, due to the phytochemicals present. *Artemia,* commonly known as brine shrimp, is a tiny halophilic invertebrate belonging to class *Crustacean*, which plays an important role in saline aquatic and marine eco-systems. Besides its usage in aquaculture, it is also highly valued for its application in toxicity detection and it is used in areas such as Ecology, Physiology, Ecotoxicology, Aquaculture and Genetics. Furthermore, *Artemia* based lethality assay (brine shrimp lethality assay, BSLA) is rapid, convenient and low cost. Presently, brine shrimp lethality assays are enormously employed in research and applied toxicology. It has been used in the study of natural products as a preliminary toxicity assay to screen a large number of extracts and compounds for drug discovery in medicinal plants. The aim of this review paper is to collect, organize, select and discuss the existing knowledge about the different uses of *Artemia salina* as a bench-top bioassay for the discovery and purification of bioactive natural products.

Keywords: *Artemia*, brine shrimp, general toxicity, natural products, correlation, alternative model, screening.

1. INTRODUCTION

Natural products contain hundreds to thousands of interrelated chemical compounds that show different biological and therapeutic effects [1, 2]. There is an increasing interest in the use of natural products from plants, animals or microorganisms for the development of new therapies for the treatment of cancer [3]. However, the management and treatment of cancer are often ineffective due to adverse reactions, drug resistance or inadequate target specificity of the anti-cancer agents [4].

About 12.5% of the 422,000 species of higher plants are known to possess medicinal properties and constitute a principal source of bioactive molecules. These figures may expect to increase as many of them have not yet been screened for their biological properties. In modern times, more than a quarter of all commercialized drugs are obtained from compounds isolated from plant extracts and many others are synthetic analogues [5].

Natural products can be a valuable source for the development of new medicines due to their varied range of biological and pharmacological activities. Nevertheless, their potential toxicity should be considered to avoid adverse side effects which are usually common. In this regard, toxicity studies should be done in parallel with pharmacological activity to ensure their safe use as potential drugs. Traditional methods of drug discovery through the assessment of the biological activities (*In-vitro* cancer cell lines, small mammals like mice) of a large number of compounds have proven to be financially costly, time-consuming and, above all, the probability of the evaluated compounds to finally succeed is low [6, 7]. The use of mammalian models in drug discovery has increased over the past decades as they provide meaningful human-like predictions and information on the mechanistic, effectiveness and toxicity of the plants studied. However, actions from non-governmental organizations (NGOs) towards protecting and preserving animals are still frequently causing restrictions on the use of animals in science mainly due to religious, social and ethical dilemmas [8]. For these reasons, other organisms have started to be used as laboratory model organisms in the search of novel bioactive compounds [9]. The best study models with minimal ethical requirements are invertebrate animal models. They may be highly imperfect, but they are quite useful in research studies and can be inferred to vertebrates. In addition, a preliminary screening in *in vitro* assays serves to prioritize only the best chemicals for further screening of vertebrates [10].

Brine shrimps, *Artemia* also commonly known as sea monkeys, are the most suitable and advantageous way to examine the biological activities of different plant extracts [11]. *Artemia spps* are broadly used in lethality studies and they are also a convenient starting point for cytotoxicity assessment, when screening for the toxicity of natural products, and is based on their ability to kill a laboratory nauplii [12].

Among other animal model assays, the brine shrimp lethality test is the most user-friendly and efficient [13, 14]. This method is habitually used as a safe approach when studying the cytotoxicity and antitumor activity of natural or synthetic compounds derived from plant extracts screening in traditional drug discovery [15]. Although there may be a decrease in the solubility of some chemical substances in the saline medium producing false positives due to the toxicity of the solvent itself, this method is cost-effective, simple, rapid, reliable, comprehensive and can be used to detect the general toxicity in a broad spectrum of natural products. The wide use of *Artemia* as a test organism is mostly because it is easy to culture and well-studied [16-24]. This review intends to evaluate and summarise the use of the *Artemia* lethality bioassay also known as brine shrimp for the general toxicity study of natural products.

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1.1. General Features of the Genus *Artemia*

Brine shrimp belongs to the *Artemia* genus (family Artemiidae, order Anostraca, subphyllum *Crustacea*), a primitive aquatic arthropod present in salt lakes [25]. It was first reported from Urmia Lake in 982 AC by an unknown Iranian geographer, and then in 1756, Schlösser pictured both sexes clearly. Linnaeus (1758) described brine shrimp as Cancer salinus but 61 years later, Leach (1819) transferred it to *Artemia salina* [26].

Generally speaking, genus *Artemia* contains six bisexual species namely, *A. franciscana, A. salina, A. sinica*, *A. urmiana, A. persimili* and *A. tibetiana and* many other parthenogenetica *Artemia* populations which are distributed all over the world [27]. *Artemia salina, Artemia franciscana* and *Artemia urmiana* are the most commonly used species for biological activity study. Nonetheless, *A. salina* is the most extensively studied of the *Artemia* species; estimated to represent over 90% of the studies in which *Artemia* is used as an experimental test organism [7]. They are very important for aquaculture because of their high nutritional value.

The brine shrimp *Artemia* is a primitive arthropod with thin chitin coated segmented body. It grows to an adult size of about 8-12mm long [28]. They are vital in the flow of energy in the aquatic food chain [29, 30] due to the fact that, they feed on microalgae and, in turn, constitute the zooplankton that is used to feed larval fishes [27, 31]. Various authors have demonstrated that the cysts and nauplii biometry and the nutritional properties of *Artemia* may largely differ from one batch to another. Also, contrasting biological, chemical and physiological features can be observed depending on the geographical regions of origin [32]. The *Artemia* nauplii provide better growth and survival rate to fish and thus, they have economic and ecological importance.

Brine shrimps have a short life cycle which makes them suitable as laboratory animal. They have a high fecundity rate, and they can be obtained from cysts hatching (Fig. **1**) [33]. Commonly, they can be found living and breeding in the sea and brackish water. *Artemia* are common in isolated environments with high salinity and relatively warm temperatures, like salt lakes, lagoons and salt mines. The prevalent attribute of all *Artemia* habitats lies in their great ability to reside in highly elevated saline conditions and in maintaining osmotic homeostasis in those conditions [29, 34]. Its habitat ranges in salinity from 5-250 gL^{-1} and in temperatures from 6 to 35°C. There are three main features that lead to their ability to survive in these hostile environments. The first is the capability to synthetize pigments that help them manage the low oxygen levels of high salinity mediums; the second is their ability to form dormant cysts when conditions become unfavourable [35, 36]; and the third is the presence of the Na^+/K^+-ATPase in specialized organs that expel salt from the isosmotic internal hemolymph to the external medium [37]. Their ability to survive in hypertonic environments is a unique mechanism used by them for defense against predators.

2. USE OF *ARTEMIA* IN BIOLOGICAL ACTIVITY STUDY

2.1. Methodology for the Brine Shrimp Lethality Bioassay

There is an increasing need to evaluate the pharmacological activity and toxicity potential of novel molecules using animal models. The choice of an adequate animal model should take into account global aspects of biology, adaptability to laboratory conditions, ecological relevance, systematic use, and life cycle [38]. However, there is a current tendency towards limiting the use of laboratory animals in toxicological tests. Due to the fact that larvae of the crustacean *Artemia* are sensitive to a variety of substances, the brine shrimp bioassay has been used as a quick and simple test for predicting the toxicity of natural product extracts, as well as compounds with biological activity [20]. Brine shrimp cysts are hatched in artificial seawater (3.5% NaCl solution) for approxi-

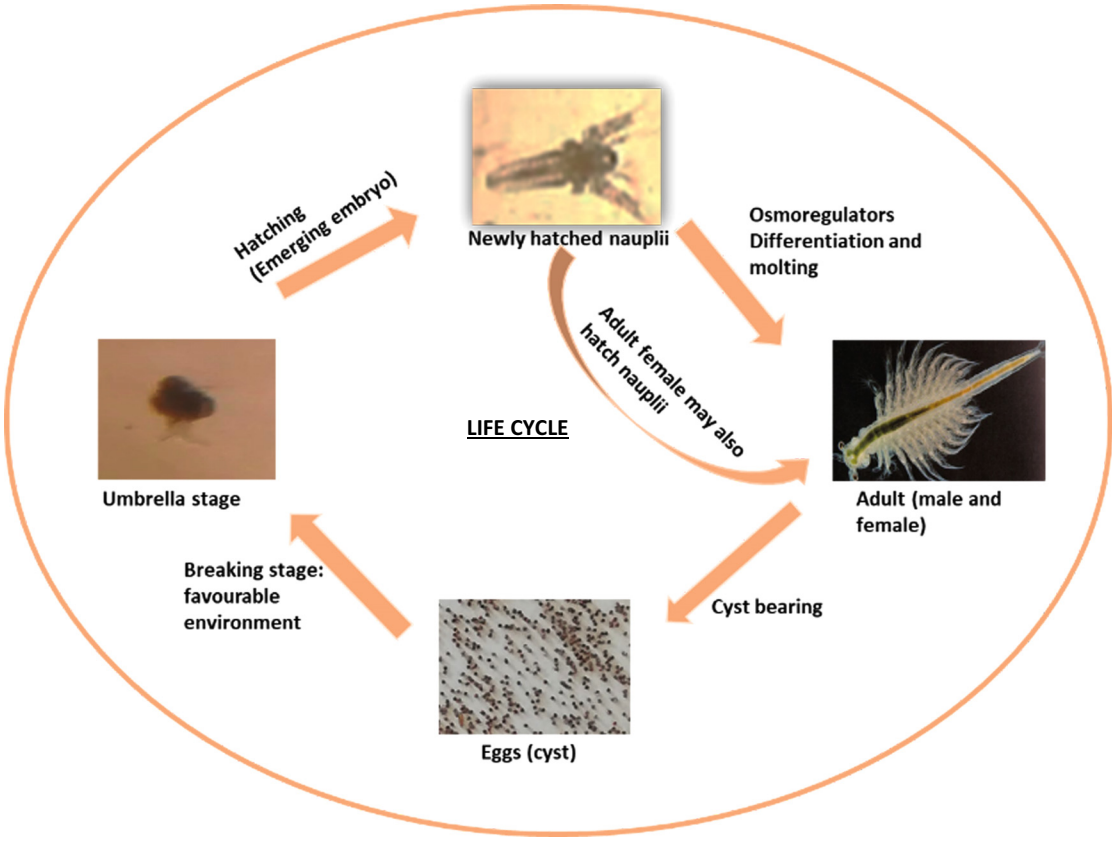

Fig. (1). Different stages of Artemia life cycle.

mately 48h under conditions of aeration, continuous illumination and temperatures of 22-29°C. Nauplii are separated from the eggs by attracting the organisms to one side of the vessel with a light source. After hatching, the active nauplii are collected with a plastic pipette for study. Next, ten to fifteen brine shrimp larvae (nauplii), which have developed for 48 hours, are transferred to each well. The test samples added, and plates are incubated at 22-29°C for 24 hours. The test sample is dissolved in DMSO (dimethyl sulfoxide) at 2% maximum to prevent possible toxicity due to this solvent [13, 39]. The plates are then examined under a binocular microscope (12.5×). After, the nauplii are killed with methanol and the number of deaths (non-mobile nauplii) in each well is counted. The percentage of death calculated and half of the lethal concentration (LC_{50}) is determined using the equation below [7, 40]. All tests should be done in triplicates.

$$(\%\text{Deaths}) = \frac{\text{Test-Control}}{\text{Control}} \times 100$$

The positive controls used in this assay are potassium dichromate, thymol, glacial acetic acid, berberine chloride and pure DMSO [16, 41-45]. According to Meyer *et al.,* crude extracts and pure substances with LC_{50} value < 1000 µg/ml are toxic and the mortality rate of brine shrimp is proportional to the concentration of the test samples.

2.2. *Artemia* as a Model Organism

Small animals are often used as a toxicological model for humans' systems in biological, medical and environmental research. Invertebrates possess a unique advantage as model organisms for biological study. Due to their simple anatomy, brief life cycle and small size, a huge number of invertebrates can be studied in a single experiment. Besides, these organisms combine genetic amenability, low cost compatible with large-scale screens. Despite these characteristics, invertebrates have a primitive organ system and do not have an adaptive immune system, giving rise to some limitations for their use in human diseases [46, 47]. On the downside, protein divergence between invertebrates and humans causes a high rate of false negatives. Regardless of these important limitations, they are still a necessary tool to close the gap between traditional *in vitro* and preclinical animal assays. Their cost of housing is less compared to animals. There is also the possibility of extrapolating research studies to vertebrates [39, 40].

Aquatic animals used to assess the toxicity of chemicals include zebrafish (*Danio rerio*), different species of Daphnia (*D. magna*, *D. pulex*), *Thamnocephalus platyurus* and *Artemia spps*. Although brine shrimp was considered to be the least sensitive organism among a group, it is better due to the simplicity of procedures and lower volumes of toxicant required to develop the test [38].

The acute fish test for example has long been used in initial toxicity testing; for this reason, a lot of information has been accumulated for existing chemicals. A closer inspection of existing acute fish LC_{50} data, however, reveals differences in orders of magnitude not only between the species, but also for the same species between laboratories. Single species LC_{50} data may thus raise doubts on the accuracy, reproducibility and, in more general terms, regarding toxicological relevance. In addition, sincere ethical concern has evolved, considering the fact that there are doubts that fish exposed to acutely toxic concentrations of chemicals suffer serious distress and pain, which is certainly not compatible with current animal welfare legislation [49].

In an attempt to solve these issues, in 2001, a working group convened by the German Standardisation Organisation (*Deutsches Institut für Normung*, DIN) came up with a protocol for an alternative assay based on zebrafish embryos as a replacement for the acute fish test in routine whole-effluent testing. In this assay, the toxicological endpoints for assessment are coagulation, failure to develop somites, lack of tail detachment from the yolk as well as lack of heartbeat after 48h of exposure after fertilization. Zebrafish only hatch after approximately 72-96h, thus this alternative method does not represent an animal test in legal terms [50]. On the contrary, several studies have shown consistent distinct patterns of toxicity among different brine shrimp strains and even geographical origin of the cyst lot. Studies have also proven genetic similarities between *Artemia* and humans e.g. the highly conserved Heat shock proteins 70 (Hsp70) family. The Hsp70 family is a group of stress response proteins that have been studied extensively in prokaryotic and eukaryotic organisms due to their potential role in pathology, *e.g.*, cancer and apoptosis. The number of Hsp70 family members expressed depends on the species. Heat shock proteins (HSPs) are a family of highly conserved proteins which respond to environmental stressors, such as high temperature, ultraviolet light, inflammation, infection and toxins [51]. Several Hsp70 gene family members (conserved Hsp70 domains: heat shock cognate 70 (HSC70); heat shock 70 kDa cognate 5, HSC70-5; heat shock 70 kDa cognate 3 (HSC70-3)/BIP/Grp78 and hypoxia up-regulated protein 1, HYOU1) have been identified in *Artemia* species as a result of its representation of the vertebrates in toxicity testing. On the other hand, Hsp70 genes existing in the human genome are also involved in the response to environmental changes in the human organism [52]. As both organisms present the same kind of genes that could be expressed in the same conditions (*e.g.* the presence of toxins or drugs), this may favour the further establishment of the link between humans and *Artemia* spp. genomes that could make it possible to extrapolate the toxicity of the test samples.

As was stated before*, Artemia spp.* is a crustacean with the ability to survive under tough conditions, living mostly in hypersaline habitats. In the evolution tree, they are closely linked to zooplankton such as copepods and Daphnia [53]. *Artemia salina* and *Artemia franciscana* are the two brine shrimps species often used in the biological activity study. They are used as test organisms in ecotoxicology, having applications in various fields as research models [48, 59] mainly due to the abundance of their cysts, availability for purchase, easy transformation from hatching into the larvae cycle and the ease to maintain in a laboratory environment as compared to other brine shrimps. In most situations, they are employed as laboratory test organisms in ecotoxicological studies to estimate short-term acute (STA) [54] and long-term chronic (LTC) toxicities [24]. This is because the molecular, cellular, and physiological levels of *Artemia* spp. are drastically altered when in contact with even slight levels of contamination [34, 43].

Artemia acute toxicity tests are known to show low sensitivity; however, prolonged exposure assays are more sensitive but require quite complex procedures that discourages their use [23]. In STA toxicity, the death rate and behavioral parameters are used to study the impact of compounds on the *Artemia* nauplii. The swimming speed, the length and the rate of death after 5-30 seconds of no visible appendage movement are monitored [56]. Furthermore, STA toxicity is highly exploited to study the consenquence of biocides on *Artemia* and their aquatic environment that could endanger the survival of other aquatic organisms. Some of these biocides include acetylcholinesterase, organochlorine, organophosphate, carbamate, pyrethroid. It also has applications in the measurement of oxidative stress parameters (glutathione-peroxidase, glutathione reductase, glutathione-S-transferases) and lipid peroxidation [57, 58]. Conversely, in LTC, the *Artemia* is observed based on its evolutionary phases from the larval phase to adulthood evaluating features such as growth, reproduction and survival after 7-28 days exposure using low concentrations of the test samples (µg/L) [11].

The bioassay methods have the advantage of being able to detect global toxicity, *i.e.*, they detect any toxic compound to which the bioindicator would be sensitive and not only a specific target toxin [59]. These bioassays also allow toxin identification and quantification using dose-response curves when pure toxin stan-

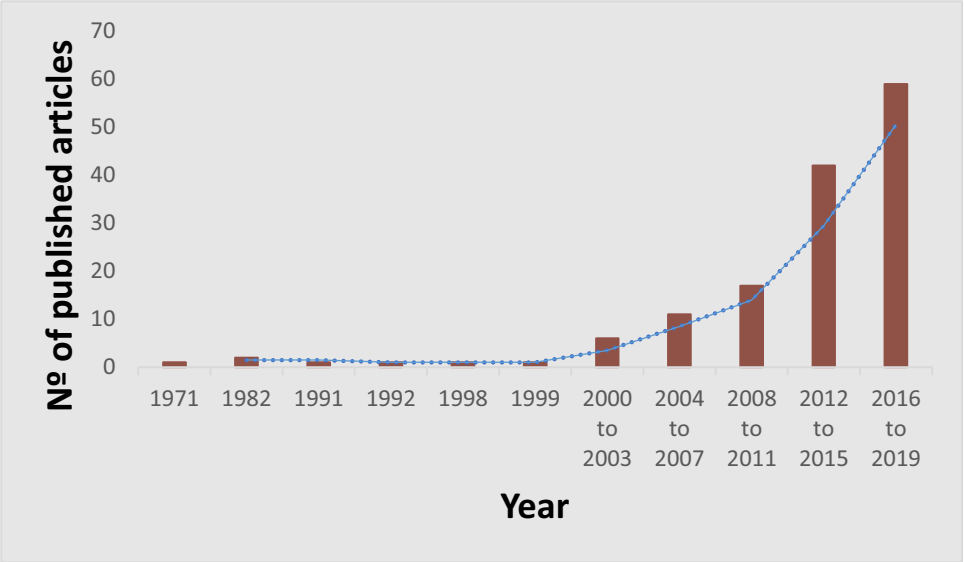

Fig. (2). Publications in which Brine shrimp is used as a screening method for general toxicity in plants from 1971 to 2019.

dards are available. They are an economic alternative and suitable for small laboratories with limited resources [16-23].

For all these reasons, *Artemia* assay has been used for several years around the world as a laboratory organism. It is used to study the cytotoxicity of medicinal plant extracts most likely toxic to shrimp larvae [16, 60-62]. It is also used to help choose the plant fraction with more potential of containing active components or to detect the possible toxicity of their constituents [63-65]. *Artemia* appear to have the capability to be suitable model species to investigate the general toxicity of medicinal plants and their possible use in drug lead compound search through laboratory experiments [66].

2.3. *Artemia* in Natural Product Chemistry

Natural products could be a good source of novel anti-multidrug resistant agents thanks to their several biological activities and wide structural divergence [67]. However, there is a great need to carry out several and diverse studies, including both pharmacological and toxicity studies, in order to make these plant-based treatments harmless. The toxic effects of plants can originate from several sources. They can come from several kinds of contaminants or the actual components of the mixture. Plants may be useful either in their crude state, advance forms or pure forms providing a source for therapeutic agents. *Artemia salina*, *Artemia franciscana*, *Artemia urmiana* and *Thamnocephalus platyurus* can serve as triage for toxic compounds *i.e.*, used for the initial screening of toxicity [16, 68]. Over the past few years, BSLA has been a useful tool for toxicity studies of different types of plant products (Fig. **2** and Table **1**) [16].

BSLA is used for the preliminary study of the general toxicity of natural products [7, 60, 119-133], compounds [155-162], heavy metals [134, 135] metal ions [136], cyanobacteria [137-139] algae [63], dental materials [55], nanoparticles [140] and to screen marine natural products. It is an indicator of potential antitumor, insecticidal, and fungicidal activity [141]. It evaluates toxicity after 24h [17, 45, 100, 144, 147, 151, 153, 160, 169] and after 48h of exposure [150]. Rodrigues *et al.* used the *Artemia salina* to determine the percentage (%) of viability of *Limonium algarvense* Erben and *Camellia sinensis* (L.) Kuntze (green tea) extracts after 48hrs of exposure. The results are expressed as the mean of three independent experiments, each with internal triplicates (n = 9) [150].

The use of *Artemia* spp to determine the general toxicity of natural products has been reported in several papers. They report using mainly *Artemia salina* [18, 44, 45, 63, 100, 118, 127, 141-167] and *Artemia franciscana* [24, 42, 43, 168].

Akhalwaya *et al.* used *Artemia franciscana* to establish the toxic concentrations for leaves, stems and essential oils of *Cissampelos torulosa* E.Mey. ex Harv. & Sond., *Clausena. anisate* (Willd.) Hook.f. ex Benth., *Croton gratissimus* Burch., *Warburgia. Salutaris* (G.Bertol.) Chiov., *Tarchonanthus camphoratus* L. and *Tetradenia riparia* (Hochst.) Codd plants traditionally used for the treatment of oral diseases. The plant extracts and essential oils were selected if they exhibited noteworthy (>1mg/mL) Minimum Inhibitory Concentration (MIC) values against any of the oral pathogens investigated in this study. In this study, BSLA revealed that the leaves, stems and essential oils are considered highly toxic with LC_{50} values of 132 µg/mL, 126 µg/mL and 194 µg/ mL, respectively. The toxicity was determined both after 24h and 48h using glacial acetic acid as a positive control [44].

Lechen plants are often sited as a source of several bioactive natural compounds. Jha *et al*. studied the effect of 84 of these plant extracts on brine shrimp live nauplii mortality after 24 h of treatment. The activity was categorized into four groups-strong (80% to 100% death of nauplii), moderate (50% to 80% death of nauplii), weak (less than 50% death of nauplii) and inactive (no death at all). Berberine chloride was used as positive control and seawater was used as negative control and these samples were toxic against the *Artemia* nauplii [45].

Noé *et al.* used *Artemia franciscana* Kellogg nauplii to study the general toxicity of the *Syzygium australe* (H.L. Wnddl. ex. Link) B. Hyland, *Syzygium luehmannii* (F. Muell.) L.A.S. Johnson, *Syzygium jambos* L. (Alston), *Terminalia ferdinandiana* Exell. and *Tasmannia lanceolata* (Poir.) A.C.Sm. used traditionally for the treatment of a variety of pathogenic diseases. The toxicity of extracts and toxins was determined after 24h of exposure. Concisely, 400 µL of artificial seawater containing 40-60 live *A. franciscana* nauplii, was added to each well of a 48 well micro-titre plate. Individual 400 µL volumes of the test samples were added to the wells and incubated at 25ºC. Negative controls (32 g/L artificial seawater; Sigma Australia) and positive controls (1 mg/mL potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) were included in all plates. The LD_{50} value was determined as the concentration of a test substance necessary to have a lethal effect on 50% of the *A. franciscana* nauplii. The results are expressed as the

Table 1. Published studies on which Brine shrimp is used as a screening method for general toxicity in plants.

Plant	Part	Endpoint of the *Artemia* Assay	Published Year	Refs.
Acacia senegal (L.) Willd.	Fruits	24h % mortality	2014	[69]
Ambrosia maritima L.	Leaves			
Ammi visnaga (L.) Lam.	Fruits			
Foeniculum vulgare Mill.	Fruits			
Nigella sativa L.	Seeds			
Sesamum indicum L.	Seeds			
Trigonella foenum-graecum L.	Seeds	24h % mortality	2012	[70]
Glycine max (L.) Merr.	Seeds			
Sesamum indicum L.	Seeds			
Tamarindus indica L.	Stem bark	24h % mortality	2011	[71]
Amphipteryngium adstringens (Schltdl.) Standl.	Stem bark	24h % mortality	2007	[72]
Arracacia tolucensis var. *multifida* (Kunth) Hemsl.	Aerial parts			
Brickellia veronicaefolia (Kunth) A.Gray	Aerial parts			
Exostema caribaeum (Jacq.) Schult.	Stem bark			
Gnaphalium spp.	Inflorescences			
Haematoxylon brasiletto H.Karst.	Stem bark			
Hintonia latiflora (Sessé & Moc. ex DC.) Bullock	Stem bark			
Hintonia standleyana Bullock	Stem bark			
Hippocratea excelsa Kunth	Roots			
Iostephane heterophylla (Cav.) Benth.	Roots			
Ligusticum porteri J.M.Coult. & Rose	Roots			
Piper sanctum (Miq.) Schltdl. ex C.DC.	Leaves			
Poliomintha longiflora A.Gray	Aerial parts			
Scaphyglottis livida (Lindl.) Schltr.	Whole plant			
Valeriana procera Kunth	Roots, rhizomes			
Quercus infectoria G.Olivier	Galls	24h and 48h % mortality	2009	[73]
Kaempferia galanga L.	Rhizomes			
Coptis chinensis Franch.	Roots			
Glycyrrhiza uralensis Fisch.	Roots			
Callistemon citrinus (Curtis) Skeels	Fruits	24h % mortality	2011	[74]
Rubus fruticosus L. ex Dierb.	Fruits	24h % mortality	2013	[75]
Coriandrum sativum L.	Essential oil	24h and 48h % mortality	2015	[76]
Thymus vulgaris L.	Essential oil			
Indigofera gerardiana Baker	Whole plant	24h % mortality	2009	[77]
Peltophorum pterocarpum (DC.) K. Heyne	Root	24h % mortality	2011	[78]

(Table 1) Contd....

Plant	Part	Endpoint of the *Artemia* Assay	Published Year	Refs.
Passiflora glandulosa Cav.	Fruit	24h % mortality	2018	[79]
Persicaria orientalis (L.) Spach	Leaves	24h % mortality	2016	[80]
Jurinea dolomiaea Boiss.	Roots	24h % mortality	2014	[81]
Asparagus gracilis Salisb.	Aerial parts			
Sida cordata (Burm.f.) Borss.Waalk.	Whole plant			
Stellaria media (L.) Vill.	Whole plant			
Chukrasia tabularis A.Juss.	Bark, leaves	24h % mortality	2016	[82]
Turraea vogelii Hook.f.	Leaves			
Psophocarpus tetragonolobus (L.) DC.	Pods	24h % mortality	2006	[83]
Vanda tessellata (Roxb.) Hook. ex G.Don	Leaves	24h % mortality	2014	[84]
Heritiera fomes Buch.-Ham.	Bark	24h % mortality	2009	[85]
Acalypha hispida Blume	Stem barks	24h % mortality	2018	[86]
Baccaurea lanceolata (Miq.) Müll. Arg				
Bischofia javanica Blume				
Codiaeun variegatum (L.) Rumph. ex A.Juss.				
Croton paniculatus Lam				
Euphorbia antiquorum L.				
Euphorbia hirta L.				
Jatropha podagrica Hook.				
Glochidion arborescens Blume				
Macaranga tanarius (L.) Müll.Arg				
Sapium baccatum Roxb.				
Parabaena sagittata Miers	Leaves	24h % mortality	2013	[87]
Adenia rumicifolia Engl. & Harms.	Leaves	24h % mortality	2001	[88]
Caesalpinia bonduc (L.) Roxb.	Roots			
Cassia occidentalis L.	Roots			
Citrus aurantifolia (Christm.) Swingle	Leaves			
Cleistopholis patens (Benth.) Engl. & Diels	Leaves			
Cnestis ferruginea Vahl ex DC.	Roots			
Momordica cissoides Planch. ex Benth.	Whole plant			
Morinda morindoides (Baker) Milne-Redh.	Roots			
Oncoba spinosa Forssk.	Seed			
Pleiocarpa mutica Benth.	Roots			
Rothmannia longiflora Salisb.	Stem bark			
Turraea heterophylla Sm.	Leafy twigs			
Uvaria chamae P.Beauv.	Roots			
Vernonia colorata (Willd.) Drake	Stem bark			

(Table 1) Contd....

Plant	Part	Endpoint of the *Artemia* Assay	Published Year	Refs.
Carissa edulis (Forssk.) Vahl	Roots	24h % mortality	2006	[89]
Neoboutonia macrocalyx Pax	Stem bark			
Acacia nilótica (L.) Delile	Stem bark			
Strychnos henningsii Gilg	Stem bark			
Azadirachta indica A.Juss.	Leaves			
Myrica salicifolia Hochst. ex A. Rich.	Stem bark			
Fagaropsis angolensis (Engl.) H.M.Gardner	Stem bark			
Zanthoxylum usambarense (Engl.) Kokwaro	Stem bark			
Harrisonia abyssinica Oliv.	Stem bark			
Withania somnifera (L.) Dunal	Stem bark			
Cola cordifolia (Cav.) R.Br.	Bark, leaves	24h % mortality	2012	[90]
Acalypha hispida (Burm.) F	Flower (essential oil)	24h % mortality	2011	[91]
Mimosa pudica L.	Stems	24h % mortality	2008	[92]
Mimosa rubicaulis Lam.	Stems			
Centaurea persica Boiss.	Aerial parts	24h % mortality	2012	[93]
Grewia trichocarpa Hochst. ex A. Rich.	Roots	24h % mortality	2014	[94]
Dichrostachys cinerea (L.) Wight et Arn.	Roots			
Tamarindus indica L.	Stem bark			
Azadirachta indica (L) Burn.	Root bark			
Acacia seyal Del.	Roots			
Azadirachta indica (L) Burm	Root bark	24h % mortality	2013	[95]
Dichrostachys cinerea (L) Wight et Arn	Roots			
Tamarindus indica L.	Stem bark			
Acacia seyal Del.	Roots			
Grewia trichocarpa Hochst ex A.Rich.	Roots			
Albizia gummifera (J.F. Gmel.) C.A. Sm.	Leaves, roots	24h % mortality	2004	[96]
Cyathula cylindrica Moq.	Leaves, roots			
Cyathula polycephala Bak.	Leaves, roots			
Pentas longiflora Oliv.	Leaves, roots			
Pittosporum lanatum Hutch. & E.A. Bruce	Leaves, roots			
Croton argyrophylloides Müll. Arg.	Aerial parts	48h % mortality	2012	[97]
Maranta arundinacea Linn.	Leaves	24h % mortality	2015	[98]
Maytenus obtusifolia Mart.	Leaves	24h % mortality	2008	[99]
Ocimum suave Willd.	Leaves	24h % mortality	2015	[100]
Plectranthus barbatus Andrews	Root barks			
Zanthoxylum chalybeum Engl.	Roots barks			

(Table 1) Contd....

Plant	Part	Endpoint of the *Artemia* Assay	Published Year	Refs.
Morus alba L.	Leaves	24h % mortality	2015	[101]
Lonchocarpus montanus A.M.G. Azevedo	Roots	24h % mortality	2007	[102]
Acacia caven (Molina) Molina	Seeds	24h % mortality	1992	[103]
Acaena argentea Ruiz & Pav.	Aerial parts			
Apium australe Thouars	Aerial parts			
Aristotelia chilensis (Molina) Stuntz	Leaves			
Baccharis linearis (Ruiz & Pav.) Pers.	Aerial parts			
Baccharis rhomboidalis J.Rémy	Aerial parts			
Buddleja globosa Hope	Leaves, stem			
Centaurium cachanlahuen (Molina) B.L.Rob.	Whole plant			
Cestrum parqui (Lam.) L'Hér.	Aerial parts			
Cryptocarya alba (Molina) Looser	Leaves, bark			
Equisetum bogotense Kunth	Whole plant			
Escallonia illinita C.Presl	Leaves, Stem			
Fabiana imbricata Ruiz & Pav.	Aerial parts			
Fuchsia magellanica Lam.	Aerial parts			
Geranium core-core Steud.	Aerial parts			
Gunnera tinctoria (Molina) Mirb.	Aerial parts			
Lapageria rosea Ruiz & Pav.	Aerial parts			
Laurelia sempervirens Tul.	Leaves			
Loasa tricolor Ker Gawl.	Aerial parts			
Lomatia ferruginea R. Br.	Aerial parts			
Luzuriaga radicans Ruiz & Pav.	Aerial parts			
Modiola caroliniana (L.) G.Don	Aerial parts			
Muehlenbeckia hastulata (Sm.) I.M.Johnst.	Whole plant			
Nertera granadensis (Mutis ex L.f.) Druce	Aerial parts			
Oxalis rosea Jacq.	Whole plant			
Plantago major L.	Aerial parts			
Relbunium hypocarpium (L.) Hemsl.	Whole plant			
Rumex crispus L.	Whole plant			
Schinus latifolius (Gillies ex Lindl.) Engl.	Bark			
Tristerix tetrandrus (Ruiz & Pav.) Mart.	Leaves, seeds			
Borago officinalis L.	Leaves	24h % mortality	2011	[104]
Lawsonia inermis L.	Leaves	24h % mortality	2014	[105]
Carissa edulis (Forssk.) Vahl	Stem bark	24h % mortality	2009	[106]
Periploca linearifolia Quart. (Dill & A.Rich)	Stem bark			
Kigelia africana (Lam.) Benth.	Leaves			

(Table 1) Contd....

Plant	Part	Endpoint of the *Artemia* Assay	Published Year	Refs.
Maytenus heterophylla (Eckl. & Zeyh.) N.Robson	Root bark	-	-	-
Microglossa pyrifolia (Lam.) Kuntze	Leaves			
Strychnos usambarensis Gilg ex Engl.	Root bark			
Strychnos henningnsii Gilg	Root bark			
Lippia javanica (Burm f.) Gaertn.	Roots			
Ocimum gratissimum L.	Seeds, leaves and stems	24h % mortality	2014	[107]
Acrostichum heterophyllum L.	Leaves	24h % mortality	2016	[108]
Buddleja thyrsoides Lam.	Leaves	24h % mortality	2012	[109]
Vernonia kotschyana Sch. Bip.exWalp.	Roots	24h % mortality	2012	[90]
Adenanthera pavonina L.	Leaves	24h % mortality	2016	[110]
Acacia mearnsii De Wild.	Bark	24h % mortality	2012	[111]
Ritchiea capparoides (Andr.) Britten var. *longipedicellata* (Glig) De Wolf	Leaves, stem bark	24h % mortality	1998	[112]
Boerhavia elegans Choisy	Whole plants	24h % mortality	2010	[113]
Solanum surattense Burm. f.				
Solanum alatum Dunal				
Prosopis juliflora (Sw.) DC.				
Ficus benghalensis L.				
Ficus carica L.				
Citrus limon (L.) Osbeck				
Morus alba L.				
Acacia farnesiana (L.) Willd.				
Citrus aurantifolia (Christm.) Swingle				
Acanthospermum hispidium DC.	Aerial part	24h % mortality	2011	[114]
Argemone mexicana L.	Whole plant			
Byrsocarpus coccineus Schumach. & Thonn.	Roots			
Croton lobatus L.	Aerial part			
Canthium setosum Hiern	Aerial part			
Dichapetalum guineense (DC.) Keay	Leaves			
Nauclea latifolia Sm.	Roots			
Pterocarpus erinaceus Poir.	Aerial part			
Schrankia leptocarpa DC.	Whole plant			
Secamone afzelii (Roem. & Schult.) K.Schum.	Aerial part			
Gynotroches axillaris Blume	Leaves	24h % mortality	2013	[115]
Ocimum gratissimum L.	Leaves	24h % mortality	2017	[116]
Iris germanica L.	Rhizomes	24h % mortality	2003	[117]
Coffea arabica L.	Cherries	24h % viability	2016	[118]
Coffea canephora Pierre ex A.Froehner				

mean of three independent experiments, each with internal triplicates (n = 9) [42].

3. CORRELATION OF *ARTEMIA SALINA* ASSAY TO OTHER TOXICITY ASSAYS

Many studies have established a positive correlation between the brine shrimp assay and mice acute oral toxicity [16, 139, 151, 169-171]. Parra *et al.* studied the acute oral toxicity of 20 Cuban plant extracts which have important pharmacological properties and are used widely by the local population using "*in vivo*" and "*in vitro*" methods. Their results showed a positive correlation ($r = 0.85$ $p < 0.05$), suggesting that BSLA is a useful alternative model for toxicological studies and predicting the general toxicity of plant extracts. Also, in the work of Hossain *et al.*, the *Cassia reniger* ethanolic extracts o had more activity in both the *Artemia salina* assay and mice acute toxicity study in both the 500 mg/Kg and 1000 mg/kg body weight dose indicating a positive correlation between the two assays [172].

Araújo *et al*. studied the general toxicity and *in vivo* antitumor activity against Sarcoma-180 of *Crataeva tapia* Bark Lectin (CrataBL). In this study, CrataBL. exhibited *A. salina* mortality with an LC_{50} of 71.73 µg/ml and as proposed by Meyer *et al.,* crude extracts and pure substances with LC_{50} value < 1000 µg/ml are toxic. In the same light, the treatment of albino Swiss mice with CrataBL (20 mg/kg) significantly ($p < 0.05$) reduced the Sarcoma 180 volume as compared to the tumor volume of the control group; for which the antitumor activity of lectin has already been described. Hence, this reinforced a good correlation between these two assays [173]. Moshi *et al*. evaluated the toxicity of *Crassocephalum vitellinum* (Benth.) S. Moore extracts in mice and BSLA having similar results. In both assays, the extract exhibited low brine shrimp toxicity and no toxicity to mice and all the animals survived to the 28 days of observation period [174]. Ali *et al*. also showed that *Rubus fruticosus* G.N.Jones used in tribal medicine had moderate toxicity in both acute mice toxicity assay and the BSLA [75]. Other studies showed equivalent results between brine shrimp-mice models [12,13], however there is a need for adequate interlaboratory validation of these methods in toxicity testing.

Cytotoxicity using cell lines requires special facilities and technical skills that are not usually available in most field-monitoring laboratories. Therefore, a convenient and reliable method as BSLA for routine screening of natural product toxicity is needed. The National Cancer Institute (NCI) revealed there is a symbolic correlation amidst the *Artemia salina* assay and the *in vitro* inhibitory growth of human tumor cell lines [175, 176]. Potent anticancer activity against human cancer cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Brine shrimp assays was also proven to be in agreement and consistent [177-179].

Rajabi *et al.* assessed the cytotoxicity of 16 natural products using two methods: the brine shrimp lethality assay and the MTT assay in L929 cells. According to the results, the correlation between the LC_{50} and IC_{50} values is significant ($R2 = 0.72$, $P = 0.000$). This means that 72% variability noted in MTT method could be accounted for the BSLA and 28% is unaccounted for due to measurement error. There was no statistically significant difference between the two assays when the results were compared with paired t-test ($P = 0.402$). Also, the comparison between LC_{50} and IC_{50} mean values was statistically analysed by the Chi-square test and the result showed that there are no differences between the two assay methods ($P = 0.235$). Thus, the results obtained by BSLA are comparable with MTT results [121].

Zengin *et al.* confirmed good biocompatibility between the general toxicity using the *A. salina* model and MTT method. They studied the toxicity of *Rubus sanctus* Schreb. and *Rubus ibericus* Juz. collected from Turkey using the BSLA. This revealed LC_{50} values in the range of 2.52-5.12 mg/mL. Due to LC_{50} values obtained, a concentration of 500 µg/mL was selected, and the cytotoxicity was evaluated on human colon cancer derived HCT 116 cell using the MTT test. The tested extracts (10-500 µg/mL) confirmed good biocompatibility, as revealed by the null effect on cell line viability in the range (10-100 µg/mL) [167].

While in the search for new molecules that exhibit selectivity for a tumor cell but not a normal cell, the work of Natalija *et al.* proved similar results. They tested compounds against the K562 leukemia cells and in the brine shrimp test. The results of the brine shrimp test were in good correlation with cytotoxicity. The five steroid dimers that exhibited the highest cytotoxic actions against tested malignant cell lines were also active in the *A salina* model with an IC_{50} in the range of 0.026-0.068 mg per disc [180].

The series of studies conducted by Mondal *et al.* on the methanolic extract from the leaves of *Colocasia affinis* Schott demonstrated a correlation between the brine shrimp assay and *in vitro* growth inhibition of human solid tumor cell lines demonstrated by the NCI [181]. More so, in 2019, Olivares-Bañuelos *et al.* studied the Hexane (Hx) and Chloroform (Chl) extracts of Brown Seaweed *Egregia menziesii* (Turner) Areschoug in both the *A. salina* assay and the MTT assay using the glioma cancer cell lines from mouse, rat and human. Their results showed that the extracts are effective by diminishing the viability of glioma cell lines. Similar results were observed in the BSLA as the Hx and Chl extracts inhibited up to 100% of *A. saline's* growing, and therefore, they must be considered as cytotoxic agents. This further affirms the possibility of extrapolating the results of BSLA to the cytotoxicity of cell lines [162]. It should be however noted that the toxic doses for *Artemia salina* are in the range of 10-100 times higher in comparison to cell culture methods [55].

In the study of Matthews, the *Artemia salina* was used as a laboratory organism to investigate the capacity of compounds to prevent superoxide-mediated toxicity. In this study, it was established that *Artemia salina* are remarkably active against the menadione bisulfite. The toxicity of this compound is believed to be seemingly mediated by intracellular superoxide generation. Hence, for this reason, this cheap, useful, user-friendly assay requiring little skills could be a good tool to evaluate and search for compounds with the ability to prevent harmful effects by superoxide or other active oxygen species [182].

In the past, the cytotoxicity of cyanobacteria could be determined using only the brine shrimp bioassay. However, contrary to the above works, Hisem *et al.* suggested that it is not enough to determine the cytotoxicity of cyanobacteria only with the brine shrimp bioassay because cytotoxicity is more a distinctive attribute in cyanobacteria in comparison with toxicity to *A. salina*. In the work reported, the murine lymphoblastic cell line Sp/2 and brine shrimp (*A. salina*) were used to screen the general toxicity of heterocystous cyanobacteria from different habitats to determine the danger presented by cyanobacteria in order to contrast these two laboratory models. It was found that just 8.6% of the tested strains were exceedingly toxic to both *A. salina* and the Sp/2 cell line, and only two of the tested strains were toxic to *A. salina* and not to the murine cell line. The authors concluded that *A. salina* toxicity test should not be used to determine the possibility of the health risk posed by cyanobacteria to humans, proposing instead that *in vitro* mammal cells be used to assess the risk of cyanobacteria [137].

The BSLA shows the general toxicity and is not peculiar to any pharmacological activity. Therefore, toxicity from this test should be treated with caution and more specific toxicology assays should be performed. This is because in a study done by Movahhedin *et al.*, regardless of the slight toxicity demonstrated by the methanolic extracts of *Caulerpa peltata* J.V.Lamouroux against the *Artemia nauplii,* this extract was almost inactive against the L5178Y mouse lymphoma cells. Thus, cytotoxic molecules with activity in the BSLA may not all be anti-cancerous [183].

4. SENSITIVITY OF *ARTEMIA SALINA* ASSAY

For the past decades, *Artemia* nauplii has been used as a tool to analyse the general toxicity in teratology screens, ecotoxicology and natural products. It has been found to have a good relationship with other model laboratory assays to detect antitumoral compounds in natural products. BSLA can be done using hatched nauplii (lethality test) based on the inhibition of hatching of the cyst (hatchability test), different age specimens and the disruptions on enzyme properties [136]. However, most researchers have made use of the lethality test. Carballo *et al.* demonstrated that natural products could be tested for their biological activity using both the BSLA and the hatchability assays with the lethality test being more sensitive [184].

Artemia salina assay is reported for a high natural death rate up to 83% as a consequence of the lack of food [11,55]. Dose-response experiments indicate that the sensitivity of *A. salina* to algae extracts and commercially available Decadienal is strongly dependent upon an increase in dose, exposure period and the particular assay chosen [185]. Comparing the relationship between an increase in concentration and lethality of the nauplii, the degree of mortality increased in a concentration-dependent manner which peaked at 1 mg/mL [186].

High death rates occur within 24h of exposure compared with the control with a significant difference of $p < 0.01$. Death may be due to abnormalities observed which included improper development of mandibles and under developed endopod, endite, and the swimming setae of the second pair antenna, which resulted in an imbalanced and irregular swimming pattern [187]. According to Unuofin *et al.*, maximum sensitivity of nauplii is achieved at the second and third instar stage and it is interpreted to be after 48 hours of incubation [186].

CONCLUSION

The use of alternative models for toxicity screening has been well accepted by the scientific community worldwide. Microcrustacean used in research are easily managed organisms and feasibly cultured in large populations using laboratory methods. One of these microcrustaceans is the *Artemia*, which has been used as a method to test the biological activity and general toxicity of different drugs, and of medicinal plant extracts. The use of *Artemia* for toxicity studies is been debated by many researchers. As previously discussed, some studies did not find a good correlation between the brine shrimp assay and other cytotoxic assays and stated that there is no significant correlation between the toxicity in cell lines and *Artemia*. On the contrary, others are of the opinion that although brine shrimp test does not provide specific information regarding drugs mechanism of action, it has a good correlation with other mammalian models. It is used to detect cytotoxicity which can be extrapolated to the cell lines, making it a good method to study new cancer treatments. Hence the use of *Artemia* as a test model should be critical as its toxicity appears to depend on the mechanism of action of the test sample. It seems that samples whose toxic effect targets various basal metabolic pathways present in the eukaryotic cell, rather than being a specific mechanism against a complex multi-cellular organism may not be active in the brine shrimp assay. Thus, it is important to note that, when using *Artemia* as a test organism, further and more specific bioassays are necessary in order to confirm these presumptions.

LIST OF ABBREVIATIONS

AC	=	After Christ
BSLA	=	Brine Shrimp Lethality Assay
BSLT	=	Brine Shrimp Lethality Test
Chl	=	Chloroform
CrataBL	=	*Crataeva tapia* Bark Lecitin
DIN	=	German Standardisation Organisation (*Deutsches Institut für Normung*)
DMSO	=	Dimethyl sulfoxide
Hx	=	Hexane
IC_{50}	=	Medium Inhibitory Concentration
$K_2Cr_2O_7$	=	Potassium Dichromate
LC_{50}	=	Medium Lethal Concentration
LD_{50}	=	Medium Lethal Dose
LTC	=	Long-Term Chronic
MIC	=	Minimum Inhibitory Concentration
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	=	Number (of samples, *etc*.)
NCI	=	National Cancer Institute
P	=	Probability
R	=	Ratio
Ref.	=	Reference(s)
R & D	=	Research & Development
STA	=	Short-Term Acute

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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