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Brine shrimp (*Artemia salina Leach*) as an alternative model for assessing the *in vivo* antioxidant activity of rutin

Artemia salina Leach como modelo alternativo para avaliação da atividade antioxidante *in vivo* da rutina

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ABSTRACT

Although there are currently more than 400 methods for assessing the antioxidant activity of natural compounds, all *in vivo* models still require the use of rodents. In this context, the use of alternative models, such as invertebrate species, has gained prominence. Here, we standardized an *in vivo* model to study the antioxidant activity of natural compounds using Brine shrimp (*Artemia salina* Leach) as an alternative to rodents. Exposure of *A. salina* nauplii to 0.1% hydrogen peroxide (H_2O_2) for 1h was considered a potential model. Herein, rutin, which possesses well-known antioxidant activity, abolished the H_2O_2-induced lethality in *A. salina* nauplii. In conclusion, the low cost, easy maintenance, and absence of the need for ethical approval make *A. salina* a promising model for *in vivo* evaluation of antioxidant compounds.

Keywords: Antioxidant; *Artemia salina* Leach; Alternative model; Natural product; Rutin; Hydrogen peroxide

RESUMO

Embora existam atualmente mais de 400 métodos para avaliar a atividade antioxidante de compostos naturais, todos os modelos *in vivo* ainda requerem o uso de roedores. Nesse contexto, o uso de modelos alternativos, como espécies de invertebrados, tem ganhado destaque. Aqui, padronizamos um modelo *in vivo* para estudar a atividade antioxidante de compostos naturais usando o *Artemia salina* Leach como alternativa aos roedores. A exposição de náuplios de *A. salina* ao peróxido de hidrogênio 0,1% (H_2O_2) por 1h foi considerado um modelo potencial. Nesse sentido, a rutina, que possui conhecida atividade antioxidante, aboliu a letalidade induzida por H_2O_2 em náuplios de *A. salina*. Em conclusão, o baixo custo, a fácil manutenção e a ausência da necessidade de aprovação ética tornam *A. salina* um modelo promissor para avaliação *in vivo* de compostos antioxidantes.

Palavras-chave: Antioxidante; *Artemia salina* Leach; Modelo alternativo; Produto natural; Rutina; Peróxido de hidrogênio

INTRODUCTION

Oxidative stress is induced by free radicals and oxidants and causes harmful effects on important cellular structures (PIZZINO et al., 2017). It occurs due to an imbalance between the production and accumulation of reactive oxygen species (ROS) in cells and tissues and the ability of a biological system to detoxify these toxic products (RADI, 2018). A large body of evidence shows that oxidative stress is responsible for the onset and/or progression of several chronic diseases, such as cancer, diabetes, metabolic disorders, atherosclerosis, arthritis, dementia, obesity, osteoporosis, and cardiovascular diseases (PIZZINO et al., 2017; RADI, 2018). Thus, the use of compounds with antioxidant properties can assist in the prevention and treatment of diseases associated with oxidative damage.

Natural products are the primary source of compounds with antioxidant activity (OLIVEIRA et al., 2018). Phenolic compounds present in plants, fruits, cereals, and bee products have a potent antioxidant effect, which is associated with inhibition of ROS production, increased expression and activity of antioxidant enzymes, induction of the natural antioxidant system, and stabilization of free radicals (BANJARNAHOR; ARTANTI, 2014; DANGLES, 2012). Therefore, methodologies aimed at screening the antioxidant capacity of natural compounds are essential for developing new antioxidant agents. Currently, more than 400 *in vitro* and *in vivo* methods are used to investigate the antioxidant effects of complex samples (ALAM et al., 2013) *so far, for evaluating antioxidant activity of various samples of research interest were gone through where 407 methods were come across, which were repeated from 29 different methods. These were classified as in vitro and in vivo methods. And those are described and discussed below in this review article. In the later part of this review article, frequency of in vitro as well as in vivo methods is analyzed with a bar diagram. Solvents are important for extracting antioxidants from natural sources. Frequency of solvents used for extraction is also portrayed and the results are discussed in this article. As per this review there are 19 in vitro methods and 10 in vivo methods that are being used for the evaluation of antioxidant activity of the sample of interest. DPPH method was found to be used mostly for the in vitro antioxidant activity evaluation purpose while LPO was found as mostly used in vivo antioxidant assay. Ethanol was with the highest frequency as solvent for extraction purpose (Alam et al., 2013)*. For all *in vivo* methods, the testing samples are usually administered to the animals (generally rodents) at a definite dosage regimen. After a pre-established period, the animals are usually killed, and their blood or tissues are analyzed (ALAM et al., 2013) *so far, for evaluating antioxidant activity of various samples of research interest were gone through where 407 methods were come across, which were repeated from 29 different methods. These were classified as in vitro and in vivo methods. And those are described and discussed below in this review article. In the later part of this review article, frequency of in vitro as well as in vivo methods is analyzed with a bar diagram. Solvents are important for extracting antioxidants from natural sources. Frequency of solvents used for extraction is also portrayed and the results are discussed in this article. As per this review there are 19 in vitro methods and 10 in vivo methods that are being used for the evaluation of antioxidant activity of the sample of interest. DPPH method was found to be used mostly for the in vitro antioxidant activity evaluation purpose while LPO was found as mostly used in vivo antioxidant assay. Ethanol was with the highest frequency as solvent for extraction purpose* (ALAM et al., 2013). However, the ethical issues surrounding the use of animals in research, such as the animal welfare concern and the rigor of ethics committees, have encouraged the development of alternative models (FERNANDES;

PEDROSO, 2017). In this context, some authors suggest the use of organisms that are not classified as protected animals (*i.e.,* shrimp and water flea larvae) as an alternative for rodent use.

Brine shrimp (*Artemia salina* Leach) is a saltwater microcrustacean used as food for ornamental fish. The eggs of this microcrustacean have a high hatching rate and are easily found in stores specialized in aquariums (FILHA et al., 2012). Due to the absence of notochord, this crustacean is exempt from authorization of ethical centers and stands out as a promising alternative model to the use of rodents. Here, we standardized an *in vivo* model of oxidative damage using *A. salina*. In addition, we evaluated the applicability of this model to screen the antioxidant capacity of rutin, a natural antioxidant.

METHODS

Reagents

Dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2; 35% v/v) (Synth, São Paulo, SP, Brazil), and rutin (Sigma-Aldrich, Frankfurt, Germany) were purchased from commercial suppliers and used without further purification. Sea salt (Blue Treasure SPS, USA) and high eclosion Brine shrimp (*A. salina* Leach) eggs (Maramar, Brazil) were purchased from a store specialized in aquariums (Aquario Show, Belo Horizonte, Brazil).

A. *salina* toxicity test using hydrogen peroxide (H_2O_2) : in vivo antioxidant method

The reversibility of hydrogen peroxide (H_2O_2) toxicity by rutin and extracts was evaluated using the *A. salina* lethality test. Encysted brine shrimp eggs were incubated in artificial seawater and exposed to a 60-W lamp and pH 8. After 48h, the nauplii (10 units) were added to each set of tubes filled with 5 mL of artificial saltwater (MEYER et al., 1982). First, we determined the concentration of H_2O_2 by exposing the nauplii to different concentrations (0.01 to 10 % v/v) of this ROS and assessing the lethality every hour up to 24h of incubation. Then, the nauplii were incubated with 0.1% peroxide for 1, 2, and 3h to determine the minimum exposure time before the treatment with antioxidants to induce and reverse oxidative damage. After each time point, rutin at 5, 50, and 500 µg/mL previously diluted in dimethyl sulfoxide (DMSO) was added to the tubes, and the lethality was assessed after 24h of the treatment (addition of rutin). The number of live nauplii was determined and compared with untreated and H_2O-treated groups. Solvent control (DMSO) was utilized in all assays.

Statistical analysis

The experiments were all realized in triplicates. Data were expressed in terms of the number of live *A. salina* nauplii as median and interquartile range. All data were evaluated using the Kruskal-Wallis test with Dunns post-test. The significance level used in the tests was 5%.

RESULTS AND DISCUSSION

Oxidative stress can be artificially induced by potent oxidizing agents, such as hydrogen peroxide (H_2O_2) (DE MATTOS et al., 2003). H_2O_2 is a low-cost, transparent liquid with a characteristic odor, non-flammable, and highly miscible in water, making it a good option for inducing oxidative damage (SCHUMB et al., 1955). Through catalysis, H_2O_2 can be converted to a hydroxyl radical (•OH), a ROS known to be highly reactive and induce substantial cell damage (PIZZINO et al., 2017). Moreover, H_2O_2 is naturally produced by eukaryotic cells during aerobic respiration, representing the oxidative conditions that occur in a biological context (PIZZINO et al., 2017; RADI, 2018). Thus, due to its chemical characteristics and biological effects, we used H_2O_2 as the oxidizing agent in the model proposed in this study.

Initially, we tested the concentration of H_2O_2 needed to induce oxidative damage in *A. salina* nauplii. The outcome considered in the analysis was the lethality of the larvae over 24h, with the lowest concentration capable of inducing 100% lethality being standardized. As shown in Table 1, the concentrations between 0.25% v/v and 10% v/v induced 100% lethality within a 6h post-exposure period. On the other hand, 0.1% v/v of H_2O_2 killed all microcrustaceans after 24h and was defined as the standard concentration for this study. The untreated nauplii remained alive, validating our experimental conditions.

Table 1: Time-related lethality of different concentrations of hydrogen peroxide against Brine Shrimp Larvae (*Artemia salina*)

H_2O_2 (% v/v)	Time							
	0.5 h	1 h	2 h	3 h	4 h	5 h	6 h	24 h
10	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
5	10 (10-10)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
2.5	10 (10-10)	1 (0.5-1)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
1	10 (10-10)	7 (7-7)	3 (2.5-3)	1 (0.5-1)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
0.5	10 (10-10)	9 (9-9)	6 (6-6)	3 (2.5-4)	1 (1-1)	0 (0-0)	0 (0-0)	0 (0-0)
0.25	10 (10-10)	10 (10-10)	10 (10-10)	9 (9-9.5)	4 (2.5-4.5)	2 (1.5-3)	0 (0-0)	0 (0-0)
0.1	**10 (10-10)**	**10 (10-10)**	**10 (10-10)**	**10 (10-10)**	**10 (9.5-10)**	**9 (8.5-9)**	**8 (8-8)**	**0 (0-0)**
0.05	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	2 (2-2)
0.01	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	8 (8-8.5)
Control	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)	10 (10-10)

All data represent the median and interquartile range (Q1-Q3) of the number of live *Artemia salina* nauplii

The lethality in this model can be associated with the cytotoxic effects of the hydroxyl radicals produced in the Fenton reaction involving H_2O_2. For instance, the effects of other free radicals, such as superoxide and nitrite, are less severe than those of hydroxyl radicals since both superoxide and nitrite can be detoxified by scavenging enzymes. In contrast, no enzyme can detoxify hydroxyl radicals, making them extremely toxic and lethal to the cells (SIES, 2017) signaling and redox regulation. Generation, transport and capture of H2O2 in biological settings as well as their biological consequences can now be addressed. The present overview focuses on recent progress on metabolic sources and sinks of H2O2 and on the role of H2O2 in redox signaling under physiological conditions (1–10 nM).

The deleterious effects of free radicals such as the hydroxyl radical depend on the time of exposure to the oxidizing agent (RADI, 2018). Thus, we subsequently defined the exposure time of *A. salina* nauplii to H_2O_2 that generates reversible lethality

through treatment with antioxidant agents. For this evaluation, we selected rutin, a common flavonoid found in many plant species and that has a powerful antioxidant effect (BANJARNAHOR; ARTANTI, 2014). According to Table 2, rutin at 5 mg/mL and 50 mg/mL significantly reduced the peroxide-induced lethality in *A. salina*. In groups exposed to peroxide for an extended period (2 and 3h), a clear concentration-dependent effect was observed, with the lethality of the model being lower in tubes exposed to a higher concentration of rutin (50 µg/mL). However, the maximum antioxidant effect was observed in nauplii pre-exposed for 1h to H_2O_2. It indicates that exposure for longer periods generates toxic effects that are difficult to reverse and, therefore, can compromise the model's ability to identify compounds with moderate antioxidant potential.

Table 2: Antioxidant effect of rutin in the H_2O_2-induced lethality model using Brine Shrimp Larvae (*Artemia salina*)

Rutin concentration	Time after exposure to 0.1% H_2O_2			Without H_2O_2 (Controls)
	1h	2h	3h	
0 µg/mL	0 (0-0) [a]	0 (0-0) [a]	0 (0-0) [a]	10 (10-10)
5 µg/mL	10 (10-10) [b]	3 (2-4) [a]	2 (2-2) [a]	10 (10-10)
50 µg/mL	10 (10-10) [b]	3 (3-4) [a]	3 (3-3) [a]	10 (10-10)
500 µg/mL*	-	-	-	5 (5-7)
Solvent control (DMSO)	-	-	-	10 (10-10)

*Toxic concentration. All data represent the median and interquartile range (Q1-Q3) of the number of live *Artemia salina* nauplii. The lethality was assessed after 24h of the treatment (addition of rutin). The data were evaluated using the Kruskal-Wallis test with Dunns post-test. Different letters indicate a statistical difference between groups within the same time interval considered ($p<0.05$)

CONCLUSION

In conclusion, exposure of *A. salina* nauplii to 0.1% H_2O_2 for 1h is an alternative model for evaluating the antioxidant potential of natural compounds. However, we emphasize that this methodology is limited to natural compounds that have low toxicity and that do not significantly alter the physicochemical properties of the water where the microcrustaceans are kept. In this direction, we showed that very high concentrations of rutin (500 µg/mL) killed the microcrustaceans, preventing the evaluation of the antioxidant effect in this model. Overall, the use of *A. salina* stands out as a good alternative model for the *in vivo* evaluation of the antioxidant activity of natural compounds. The low cost, easy maintenance, and absence of the need for ethical approval make this model an attractive methodology for evaluating compounds with the ability to reduce oxidative damage *in vivo*.

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CONFLICTS OF INTEREST

None of the authors has any conflict of interest to disclose.

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