JJBS

Volume 17, Number 4,December 2024
ISSN 1995-6673
Pages 637 – 643
https://doi.org/10.54319/jjbs/170407

Jordan Journal of Biological Sciences

An *in vitro* Study into Antioxidant, Antibacterial, and Toxicity Impacts of *Artocarpus altilis* Leaf Extract

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Received: November 4, 2023; Revised: May 19, 2024; Accepted: May 25, 2024

Abstract

Artocarpus altilis (Parkinson) Fosberg, which was first discovered in 1769, mainly grows in subtropical and tropical areas. This plant is famous for having multiple applications in Asia, where people use fruits for food, leaves for drinks, and trunks for wood. *A. altilis* is also famous for treating human diseases such as diarrhea, dysentery, and other intestinal diseases. Various phytochemicals are determined in this plant and predicted to create biological activities. The bio-effects of the leaf extract were evaluated in order to supply scientific evidence contributing to this plant into the orthodox medical application. A free radical scavenging assay, a reducing power assay, a broth-diluted assay, and a brine shrimp lethality assay were performed to evaluate the extract's antioxidant, antibacterial, and toxicity capacities. The extract was clarified to be able to scavenge the free radical DPPH (EC50 = 48.65 ± 1.86 µg/mL) and active ABTS (EC = 29.27 ± 4.38 µg/mL) as well as reduce the Fe^{3+} into Fe^{2+} in solution. Moreover, the anti-positive gram bacteria were detected as an activity of the extract, also causing death to the brine shrimp with an LC50 of 87.68 ± 4.67 µg/mL. Hence, the bio-activities of the *A. altilis* extract were initially demonstrated to inhibit Gram-positive microorganisms and balance the activity of redox.

Keywords: *Artocarpus altilis*, antibacterial, shrimp lethality, antioxidants.

1. Introduction

Moraceae is a family with 60 genera consisting of over 1400 species, mainly growing in the tropical and subtropical areas of Asia, in which the genus *Artocarpus* is widely used in folk remedies (Sikarwar et al., 2014; Jagtap and Bapat, 2010). Plenty *Artocarpus* members have been proven to have the potential to treat inflammation, skin diseases, blood pressure, diabetes, etc. (Jagtap and Bapat, 2010; Tiraravesit et al., 2015; Adewole and Ojewole, 2007; Juliastuti et al., 2017). *Artocarpus altilis* (Parkinson) Fosberg is one of the standout members of the genus due to its phytochemical diversity, which includes over 50 species mainly distributed in the subtropical and tropical areas, including South Asia, Southeast Asia, Northern Australia, and Central America (Sikarwar et al., 2014; Aliefman, 2010). Sydney Parkinson discovered this plant in 1769 when he was on James Cook's first voyage (Ferrer-Gallego and Boisset, 2018). In 1773, this plant was named *Sitodium altile* and trustee at the Natural History Museum, London (Ferrer-Gallego and Boisset, 2018; Ragone et al., 1997). *Artocarpus altilis* (Parkinson) Fosberg has been officially used since 1941 (F. R. Fosberg and Swinggle, 1941). In folk therapy, each part of the plant serves as a different remedy. For example, the resin was used for the treatment of sprain, sciatica pain, diarrhea, dysentery, and other stomach diseases. Meanwhile, the bark was used for headaches, and the root was used as a purgative drug (Ragone et al., 1997). Additionally, *A. altilis* leaves are the most widely used folk remedies for inflammatory diseases (Fakhrudin et al., 2015). However, using leaves of this plant for medicine is only at the "word of mouth" level and has not been official yet despite the recorded therapeutic effects, especially the antibacterial ability. Bacterial infections are the most common threat to human health, especially those with solid toxicity or drug resistance (Mancuso et al., 2021). The problem of bacterial drug resistance has become complicated, causing many health and economic consequences despite many WHO and national policies (Zaman et al., 2017). According to WHO, although Ciprofloxacin is one of the most common antibiotics used, its restriction is predicted to rise to 93% in *Escherichia coli* and 80% in *Klebsiella pneumoniae*. In 2019, Methicillin-Resistant *Staphylococcus aureus* infection in the bloodstream was reported in 25 countries while the number recorded on *E. coli* was 49 countries. Thus, research for new bacterial therapeutics has become important, and many proposals were suggested, such as phage therapy, and modulating the microbiome in a situation where antibiotics gradually lose their effectiveness (Hauser et al., 2016). Several plant extracts are also considered a source of materials of interest in the process of finding novel treatments for bacteria (Deka and

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Jha, 2018; Adaramola et al., 2018). Most secondary compounds exhibit antibacterial and antioxidant activity (Tungmunnithum et al., 2018). The diversity of medical compounds in *A. altilis* was recorded to be related to human healthcare (Sikarwar et al., 2014). *A. altilis* was reported for its antibacterial capacity on different strains of bacteria and fungi (Qamar et al., 2014; Pradhan et al., 2013; Riasari et al., 2017a). In addition, essential compounds with promising biological activities can be found in this plant, including antioxidant activity (Tungmunnithum et al., 2018). A comprehensive investigation of the inherent bio-activities of *A. altilis* leaf extract can contribute authentic scientific evidence to guide the safe and legitimate use of this plant in medicine. To achieve this aim, a synthetic investigation of the biological activities of *A.altilis* leaf extract was investigated to illustrate the antioxidant, antibacterial, and poisonous features.

2. Materials and methods

2.1. Herbal material and extract preparation

Artocarpus altilis (Parkinson) Fosberg leaves (Voucher No. BD-2018-1050) were collected from downtown Thu Dau Mot, Binh Duong, Vietnam. The intact leaves were rinsed with distilled water. The leaves were further dried in an oven at a stable 40^{o}C until the weight was unchanged. The herbal powder was formed by grinding it into flour. The plant extract was macerated for a week with absolute methanol. The 200 mg/mL stock solution was obtained by sterile filtering, removing solvents from samples by evaporation, and dissolving them into dimethyl sulfoxide (DMSO, Merck, Germany). The *A. altislis* leaf extract (abbreviated as AE) was stored at -20°C until use.

2.2. Antioxidant capacity evaluation

2.2.1. DPPH assay

The antioxidant ability of the AE was recorded through the capacity of free radical scavenging measurements (Ly et al., 2019; Ponnusamy and Pramadas, 2011). The *A. altilis* extract ranging from 0 to 200 µg/mL was mixed with α, α-diphenyl-β-picrylhydrazyl (DPPH, Sigma-Aldrich, USA) 0.3 mM at a proportion of 1:1 (v/v) then incubated at room temperature for 30 min in the dark. The reacted solution was recorded for absorbance at 517 nm. The reactions of vitamin C and solvent to DPPH were positive and negative controls. The percentage of DPPH radical scavenging was measured as the proportion of the discoloured DPPH and the initial DPPH.

2.2.2. ABTS assay

2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS, Sigma-Aldrich, USA) 7 mM solution was reacted with 2,45 mM $K_2S_2O_8$ solution at a ratio of 1:1 at room temperature in a dark room for 14 h to form active ABTS cation radical (Ly et al., 2019). The active ABTS solution was standardized with methanol to get a reagent with an absorbance at 734 nm of 7.00 ± 0.02. The *A. altilis* extract (0 to 200 µg/mL) was mixed with the standardized ABTS at a ratio of 1:1 (v/v), and then the mixture was left in the dark at room temperature for 7 minutes. The reacted solution was recorded for absorbance at 734 nm. The solvent and vitamin C reactions to ABTS were negative and positive controls, respectively. The percentage of ABTS radical scavenging was calculated as the proportion of the original ABTS and the initial ABTS. The non-linear regression was analyzed to calculate the extract's half-maximal effective concentration (EC50).

2.2.3. Potassium ferricyanide reducing antioxidant power (PFRAP) assay

The reduction capacity indirectly demonstrates the antioxidant ability of the AE. The PFRAP assay was used to indicate the reducing power of *A. altilis* extract by using the modified method (Ly et al., 2019). A volume of 1 mL of extract was added to 2.5 mL of PBS (phosphate buffered saline, TBR Technology CO., Vietnam) at pH 6.6 and mixed with 2.5 mL of potassium ferricyanide 1% (Sigma-Aldrich, USA). The mixture was vortexed and heated at 50°C for 20 minutes before adding a volume of 2.5 mL of 10% trichloroacetic acid (Sigma-Aldrich, USA). After a 10-minute incubation, 2.5 mL of the supernatant layer was collected and mixed in 2.5 mL of water supplemented with 0.5 ml of 0.1% $FeCl_3$ (Sigma-Aldrich, USA). The blue-green colour of the solution was computed by the absorbance at 700 nm. The extract at 0 to 1600 µg/mL concentration was tested; Vitamin C was a positive control.

2.3. Pathogenic bacterial lethality

The agar diffusion assay was carried out on selected pathogenic bacteria to determine the antibacterial effect of AE, including *Escherichia coli* (ATCC 25922 and ATCC 8739), *Proteus mirabilis* (ATCC 25933), and *Salmonella enterica* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923 and ATCC 6538), *Rhodococcus equi* (ATCC 6939), *Listeria monocytogenes* (ATCC 13932). The microorganisms used in this study were derived from the American Type Culture Collection (ATCC, USA). The biomass was activated in TSA (Tryptic Soy Agar, HiMedia, India) at 37^{o}C overnight for analysis. Bacterial concentrations at 10^8 CFU/mL were regressed using the McFarland 0.5 method (Leber, 2016). Biomass was diluted with saline to obtain a density of 10^5 CFU/mL for the experiments. 100 µL of 10^5 CFU/mL spread on Muller-Hinton Agar plates, and then 6 mm wells were created using a punch stopper perforator for 50 µL AE extract loading. After a 20-hour incubation at 37^{o}C, the inhibition zones reflected the difference of the observed diameter and 6mm of the well.

The AE-sensitive strains were further detected for MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) (Ly et al., 2019). To demonstrate the MIC of the extract, the biomass of bacteria at 10^5 CFU/mL in Mueller-Hinton broth (MHB; HiMedia, India) was exposed to the AE at different concentrations, and the results were recorded by resazurin 0.02 % (Merck, Germany) supplementation. The MIC values were the most diluted AE concentration in which the biomass solution was still in blue. The treated biomass was further spread onto Mueller-Hinton Agar to clarify the MBC, which was considered as the lack of colonies' expression.

2.4. Toxicity of the extract investigation

The brine shrimp lethality assay indicated the toxicity of the extract, and the assay followed the reported description with a slight modification (Banti and

Hadjikakou, 2021). An amount of 1 mg of dried cysts (*Artemia nauplii*) was incubated in a hatcher with aeration under continuous light for egg hatching. The shrimps were cultured in separate test cups containing 2 mL of buffer saline; thirty individuals were used for a test. The larvae were exposed to the extract at a concentration of 0 to 1000 µg/mL with aeration under continuous light and without feeding for 24 hours. The solvent was a control. The larvae without movement during the 10 seconds of observation were considered dead. The survival rate was determined as a proportion of the number of alive in the test and control groups. The non-linear regression analysis was built up to indicate the median lethal dose (LD_{50}) of the extract on *A. nauplii*.

2.5. Data analysis

All of the experiments were performed at least three times. The GraphPad Prism software v.9.0.0 was used for data analysis, which was expressed as mean ± standard deviation. The differences were computed for the statistical significance at a p-value less than 0.05.

3. Results

The AE was extracted independently with 200 initial herbal powders. The extraction yield was recorded as 11.74 ± 0.04 %. The phytochemical content of AE extract was reported previously in the presence of polyphenols, especially tannins and flavonoids, cardiac glycoside, reducing sugar, and organic acid compounds (Chi, 2022a). The total polyphenol content was determined to be 13.57 ± 1.88 mg/100g dry weight, with the standard being Gallic acid. Meanwhile, the total flavonoid content was 29.64 ± 7.93 mg/100g dry weight, with the standard being Quercetin (Chi, 2022a)

3.1. The antioxidant ability of the AE

The antioxidant capacity of the extract is illustrated through its ability to scavenge free radicals and reduce redox reactions. The results showed that the AE and vitamin C scavenging effects, both at the concentration of 200 µg/mL, were not statistically different, with about 90% capturing DPPH radical (Figure 1A). The oxidizing radical scavenging capacity of the AE was recorded to be less active than that of the positive control, vitamin C. The activity of vitamin C reached saturation at a dose of 50 µg/mL and maintained this state at higher concentrations. The free radical scavenging efficiency of the AE observed on the ABTS gave similar results to the DPPH. However, the antioxidant activity of the extract tested on ABTS radical tended to be more effective than that on DPPH. For example, at a 100 µg/mL concentration, the ABTS cation scavenging capacity peaked while more than 75% of the DPPH radical was trapped (Figure 1B). In this assay, the effect of the extract reached the peak of 100% asymptotically from the concentration of 100 µg/mL to the higher concentration, and the saturation state was set up at higher coordinates than the positive control. The percentages of DPPH and active ABTS radicals were recorded to decline in an AE dose-dependent manner. The EC50 of the AE was calculated as 48.65 ± 1.86 µg/mL for DPPH and 29.27 ± 4.38 µg/mL for ABTS.

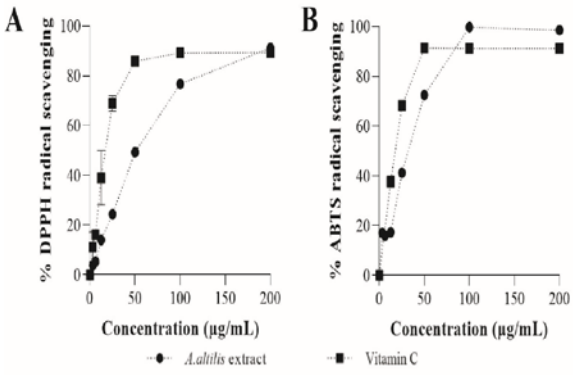

Figure 1. The antioxidant capacity of the *Artocarpus altilis* extract expressed through the free radical scavenging

The PFRAP assay was performed to assess the reducing power of AE, indirectly illustrating the antioxidant capacity (Cheng and Li, 2004). By reducing the Fe^{3+} solution into a Fe^{2+} solution with a maximum absorbing wavelength of 700nm, a curve for the extract's reduction potential was constructed to reflect the electron donor capacity (Ponnusamy and Pramadas, 2011). As illustrated in Figure 2, the reducing power of the extract was clearly shown through the gradual inflating of the amount of Fe^{2+} with the increasing AE concentration. However, the reducing power of the AE was observed to be much less effective than that of positive control.

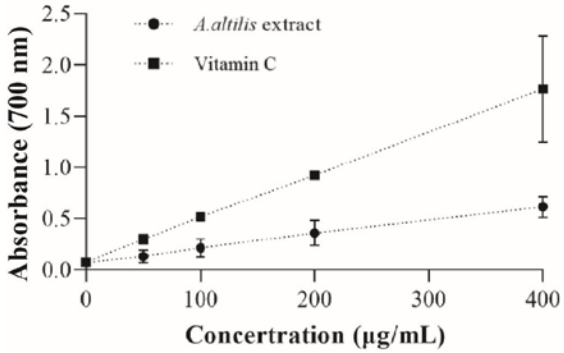

Figure. 2. The antioxidant capacity of the Artocarpus altilis extract expressed through reducing power

3.2. AE inhibited gram-positive microorganisms.

The different diameters of inhibitory zones on tested bacteria are shown in Table 1. The effect was only observed on gram-positive bacteria, with an approximate range of inhibition diameter (mm) of 5–8 for *S. aureus* ATCC 25923, 5.7–9 for *S. aureus* ATCC 6538, 8–10.5 for *R. equi* ATCC 6939; and 5.5–7.6 for *L. monocytogenes* ATCC 13932. One-way ANOVA analysis and the post-hoc Tukey's range analysis were performed and indicated that the AE showed the most potent effect on *R. equi* ATCC 6939, P-value = 0.0003.

In Figure 3, the increasing zone diameter of inhibition with increasing concentration alluded to the dose-dependent manner of the effect. This study used disk diffusion assay as a primary screening method for bacterial susceptibility, followed by the broth dilution method as a secondary screening assay. The affected bacterial strains were further analyzed for MIC and MBC values. The MIC and MBC values are detailed in Table 2. The MIC of the AE ranged from 0.1 mg/mL to 0.39 mg/mL, while the MBC was from 0.2 mg/mL to 0.78 mg/mL.

Table 1: Antimicrobial ability of the *Artocarpus altilis* extract expressed as inhibition zones

Organism	Extract concentration (mg/ml) – Inhibition zone (mm)			
	0	12.5	50	200
S.aureus ATCC 25923	-	5.01 ± 0.16	5.76 ± 0.04	7.96 ±0.49
S.aureus ATCC 6538	-	5.70 ± 0.37	6.70 ± 0.19	8.95 ± 0.13
R.equi ATCC 6939	-	8.04 ± 0.10	9.04 ± 0.04	10.46 ± 0.20
L.monocytogenes ATCC 13932	-	5.59 ± 0.25	6.79 ± 0.12	7.56 ± 0.21
E.coli ATCC 8739	-	-	-	-
E.coli ATCC 25922	-	-	-	-
P.mirabilis ATCC 25933	-	-	-	-
S.enterica ATCC 14028	-	-	-	-

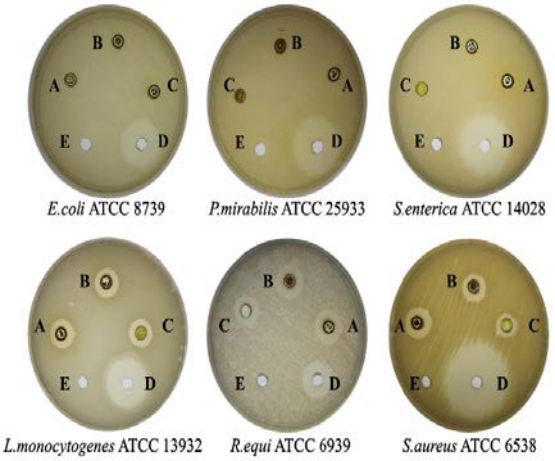

Figure. 3. The zone diameter of inhibition on various pathogenic bacteria of the Artocarpus altilis extract

Table 2. The MIC and MBC of the *Artocarpus altilis* extract and ampicillin

Organism	The AE extract (mg/mL)		Ampicillin (µg/ml)	
	MIC	MBC	MIC	MBC
S.aureus ATCC 6538	0.39	0.78	3.13	3.13
S.aureus ATCC 25923	0.39	0.39	6.25	12.50
R.equi ATCC 6939	0.10	0.20	3.13	6.25
L.monocytogenes ATCC 13932	0.10	0.20	3.13	6.25

3.3. The toxicity of AE on brine shrimp

The extract toxicity was investigated by adding the extract in different concentrations to the biomass of *A. nauplii* and then determining the percentage of mortality (Figure 4). The results showed that dead larvae increased dose-dependent and peaked at 400 µg/mL extract concentration. The EA exhibited a decisive lethality with an LD_{50} value on *A. altilis* of 87.68 ± 4.67 µg/mL.

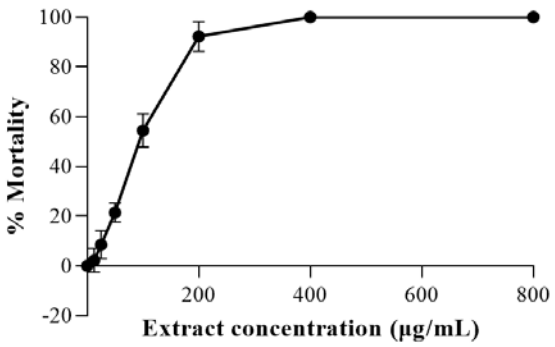

Figure. 4. The lethality effect of the Artocarpus altilis extract on brine shrimp A. nauplii after 24 hours of exposure

4. Discussion

The phytochemical content of AE was previously published at the total phenolic and flavonoid content level at 13.57 ± 1.88 mg GAE/100 g.d.w. (mg/100 g gallic acid equivalent in dried weight) and 29.64 ± 7.93 mg QE/100 g.d.w. (mg/100 g quercetin equivalent in dried weight), respectively (Chi, 2022b). The highest flavonoid content was reported to belong to the seed extract of *A. altilis* (400.86 ± 40.33 mg/100g catechin equivalent), followed by the leaf extract (334.13 ± 11.38 mg/100g catechin equivalent) (Yoanes Maria Vianney et al., 2020). Many scientific reports illustrate that antioxidant capacity mainly comes from the phenolic derived from plants, especially fruits and vegetables, instead of ascorbic acid, as popular belief holds (Wang et al., 1996). The antioxidant properties of the phenolics are formed by electron H-atom transfer, which is present in the structure (Tungmunnithum et al., 2018). The radical-induced neutralization of hydroxyls helps balance the redox in the cell (Lobo et al., 2010). The concept of free radicals, proposed by Denham Harman, was first mentioned in the *Journal of Gerontology* in 1956, suggesting that endogenous free radicals were derived from cellular activity or specifically as by-products of enzyme redox reactions (Harman, 1956). Thus far, many studies have shown the negative impact of free radicals on cellular components, namely DNA, proteins, and lipids, leading to the development of chronic human diseases, namely degenerative neurological diseases, heart-related diseases, and cancer (Sharifi-Rad et al., 2020). Maintaining redox balance helps cells avoid unwanted damage caused by free radicals, which are caused by reducing elements known as antioxidant agents (Lobo et al., 2010). The antioxidant capacity was indirectly depicted through the *in vitro* reduction of free radicals such as DPPH or ABTS of the AE. The antioxidant effect of the AE in this study was more effective than the previous research, showing the EC50 values of *A. altilis* leaf extract in DPPH assays were 140.54 µg/ml and 66.60 µg/ml (with 10% tamarind leaf co-combination) (Devi et al., 2019). In addition, the antioxidants of the other parts of *A. altilis* were also investigated, such as the fruit pulp extract, with an EC50 of 55 ± 5.89 µg/mL (Jalal et al., 2015). The AE showed an approximate antioxidant effect for the relative species, including jackfruit (*Artocarpus heterophyllus*) (Devanandan et al., 2016). The experiments with vitamin C specified the EC50 at 16.26 ± 1.54 µg/ml and 16.28 ±

1.39 µg/ml for DPPH and ABTS, which followed a similar curve to the previous report (Ly et al., 2019). The asymptote in free radical scavenging action between AE and Vitamin C shows the potential to exploit AE as a source of antioxidant materials. The donation of free electrons is also recorded through the reducing property of the extract; the higher the electron donation is, the greater is the observed reducing property (Zhong and Shahidi, 2015). The results reflect the high reducing power of AE when the extract concentration is high and vice versa. However, AE's reducing power was recorded as unrelated to radical scavenging, where there was an enormous difference between the reduction of AE and vitamin C.

In another aspect, the ability of AE to inhibit bacterial growth was observed by inhibition rings where the AE extract diffused. No inhibitory zones were observed in the Gram-negative bacterial experiments, indicating that the AE effect on these tested Gram-negative was negligible. The presence of the outer membrane and the distinction of gram-negative from gram-positive bacteria hinder the hydrophilic agents' access and permeation (Breijyeh et al., 2020). Therefore, the drug resistance in gram-negative bacteria is more conspicuous than in gram-positive (Breijyeh et al., 2020). The bias in the impact on the Gram-positive and the Gram-negative was reflected in previous reports (Shadid, 2018; Alhumaid et al., 2021).

The response to ampicillin of the tested bacteria was also investigated, and the MIC and MBC values were recorded. The anti-bacterial activity of an extract is determined by the growth-retarding and lethality of the extract against bacteria (Wallace, 2004). By comparing the MIC index of ampicillin with previous studies, it was shown that the microorganisms had average growth during the experiment (Ly et al., 2019). The antibacterial potential of A. altilis was previously reported by using agar diffusion experiments on fungi ***c***andida albicans; gram-positive bacteria streptococcus mutans**,** propionibacterium acnes, *s*taphylococcus epidermidis, *and* staphylococcus aureus; and gram-negative bacteria *p*seudomonas aeruginosa**,** escherichia coli (Riasari et al., 2017b). The biological statuses of the leaves of A. altilis, such as green leaves, yellow leaves, and fallen leaves, also influenced antimicrobial activity; fermented green leaves were more effective than the others (Riasari et al., 2017b). In fact, according to folk remedies, the fallen leaves of *A. altilis* are usually steeped in hot water to drink, cooling the liver.

In contrast, the green leaves are exploited to cure inflammatory diseases. The ability to decrease the growth of Gram-positive bacteria at low doses suggests the possibility of exploiting AE in treating bacterial diseases. Furthermore, the safety of *A. altilis* leaf extract has been reported previously, promising a potential material for extensive antimicrobial research. The investigation of the bio-effects of AE extract was tested simply by experimenting with a brine shrimp viability assay (Sarah et al., 2017). Because the shrimp can live for up to 48 hours without being fed, the experimental time of 24 hours was appropriate and ensured the vitality of the larvae (Carballo et al., 2002). The LC50 of this assay worked as a preliminary evaluation of the bioactive components as well as the general toxicity indicator of an extract (Wakawa, 2017). The LD_{50} value of less than 250 µg/mL for crude extract and less than 40 µg/mL for pure compound was considered significantly active (Rieser et al., 1996). Moreover, the safety of the A. altilis aqueous and 80% of the leaf and stem methanol extracts were also investigated on the Wistar rat model, which did not show any negative influences on the experimental host (Sairam and Urooj, 2014). The effect on brine shrimp reinforces the strong biological potential of AE extract, which is recognized as a worthy object for further in-depth studies for academic purposes and application.

5. Conclusion

The investigation into the biological activities of AE extract depicted the potent free radical scavenging capability compared to vitamin C but has a weak reducing power in a dose-dependent manner. Besides, a potent activity against Gram-positive bacteria was also recorded, notably against *R. equi* and *L. monocytogenes* with MIC values of about 100 µg/mL. The extract did not affect Gram-negative evaluated strains. Moreover, the ability to kill 50% of brine shrimp at concentrations below 100 µg/mL also confirmed the bio-effects of AE extract. Therefore, it is necessary to consider the leaves of A. altilis as a potential source of research that can be well exploited for medical applications.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

No application

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