Iranian Journal of Fisheries Sciences 21(6) 1417-1429 2022
DOI: 10.22092/ijfs.2022.128221

Research Article

The effects of razor clam (*Solen vagina*) acetone extract on immunity parameters and bacterial disease resistance in *Litopenaeus vannamei*

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Received: February 2021 Accepted: June 2022

Abstract

Bacterial infections are major limiting factors in shrimp culture that lead to great economic losses to the farmers. This study aimed to investigate the effects of *Solen vagina* acetone extract (AE) on some immunity parameters and bacterial infection by *Vibrio harveyi* in *Litopenaeus vannamei*. 5000 *L. vannamei* postlarvae were exposed to five concentrations (100, 150, 200, 250, and 300 mg/L) of *S. vagina* in triplicates for 3 weeks. A positive control (exposed to the bacteria without any AE) and a negative control (without bacteria and AE) were also considered in this study. Each group contained 600 post larvae. The highest mortality was observed in the groups with concentrations of 100 and 150 mg/L AE and the lowest was observed at a concentration of 250 mg/L AE. None of the concentrations had a significant effect on the physical and chemical properties of water. According to the histopathology analyses, the exposed post larvae showed deformation symptoms in hepatopancreas cells. Based on the results, AE could reduce and prevent mortality caused by *Vibrio harveyi*. Also, the minimum inhibitory concentration of AE for *V. harveyi* was 200 mg/L.

Keywords: Acetone Extract, *Solen vagina*, *Litopenaeus vannamei*, *Vibrio harveyi*, Razor clam

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Introduction

Global aquaculture production has been a constant and rapid growth since the 1950s (Adams and Boopathy, 2013). *Litopenaeus vannamei* is the most extensively cultivated shrimp species due to its rapid growth rate and resistance to some specific diseases (Jung *et al*., 2012; Emerenciano *et al*., 2022). Bacterial infections are major limiting factors in shrimp culture that lead to great economic losses (Adams and Boopathy, 2013). Aquatic diseases and their health problems are some of the main challenges in aquaculture production. especially in the shrimp farming industry. The major bacterial disease in shrimp aquaculture is vibriosis (Lightner, 2005). *Vibrio harveyi* is the main pathogen of the genus *Vibrio*, which under favorable conditions (stressful conditions in shrimp) can affect shrimp of the panacea family (Adams and Boopathy, 2013). *V. harveyi* pathogenicity is dependent on the bacterial strain and indicates a synergistic interaction between individual and associated factors, namely hydrophobicity, biofilm formation, survival in fish skin mucosa, serum, proteolytic, hemolytic, and cytotoxicity of Eosinophil Cationic Proteins (Darshanee Ruwandeepika *et al*., 2012; Zhou *et al*., 2012). Bacterial density is affected by nutrients, temperature, osmotic resistance, pH, and oxygen concentration, which under optimum conditions causes maximum growth of species and strains (Beste *et al*., 2007). Bacteria as an opportunistic pathogen can lead to the death of aquatic species due to sudden changes in temperature and salinity (Lightner and Redman, 1998; Vaseeharan and Ramasamy, 2003). Over the past two decades, massive shrimp losses have been reported in the hatcheries and ponds (Lavilla-pitogo, 1998; Vandenberghe *et al*., 1999; Chrisolite *et al*., 2008; Uma *et al*., 2008).

Many metabolites have been isolated from marine animals by unusual structures and biological activities. Some of these bioactive metabolites have potential bio-medical purposes. Bioactive metabolites have been isolated mostly from marine sponges, jellyfish, sea anemones, corals, mosses, mollusks, echinoderms, tunicates, and crustaceans (Bhakuni and Rawat, 2005). Organic carbohydrates and acids are found in all plants, animals, bacteria, fungi, and yeasts and the roles of these compounds in metabolic processes are essential (Buchanan, 2004).

Mollusks are widely distributed around the world, and many species live in marine ecosystems and estuaries. Among marine invertebrates, mollusks are the best source of bioactive metabolites. Bioactive compounds extracted from many classes of mollusks have antibacterial properties (Anand *et al*., 1997; Kiran *et al*., 2014). So far, several studies have been performed on the effect of extracted biomaterials on fish and shrimp (Wouter *et al*., 2005; Huang *et al*., 2006; Hoa *et al*., 2009; Huynh *et al*., 2011; Manilal *et al*., 2012; Kiran *et al*., 2014; Eswar *et al*., 2014; Huang *et al*., 2020; Torpee *et al*., 2021). Therefore, this study aimed to investigate the effect of razor clam (*Solen vagina*) acetone extract on immunity factors and bacterial diseases in western white shrimp (*Litopenaeus vannamei*) postlarvae.

Materials and methods

Sample collection

S. vagina samples were collected from the coast of Delvar located in Tangestan County, Bushehr, Iran. The samples were rinsed with sterile seawater to remove debris. Then, the shells were broken and the soft bodies were removed. The samples were then cut into small pieces and dried by air for 72 hours to separate the moisture content (Dhinkaran and Lipton, 2012).

Preparation of acetone extract (AE)

The extracted samples contained 10 g of tissue and 5 ml of acetone which was then homogenized. Tissues were sonicated for 30 min to rapidly break-down tissues and increase molecular interactions. The cell contents were released by a cell membrane disintegration. The samples were centrifuged for 45 min and the supernatants were collected and stored at -80°C (Isaac Dinkaran and Lipton, 2012). The obtained AE with five concentrations of 100, 150, 200, 250, and 300 mg/L on Muller- Hinton Broth with 2% NaCl for the assessment of MIC.

MIC method

MIC method was used to determine the lowest concentration of *S. vagina* AE to inhibit bacterial growth. Five concentrations of *S. vagina* AE (100,150, 200, 250, and 300 mg/L) were individually added to the growth medium (Muller Hinton Broth) in test tubes. Each tube contained 9 ml of growth medium and 1 ml of *S. vagina* AE. There were 10 dilutions of each concentration. These tubes were inoculated with *V. harveyi* (1×10^6 CFU/mL per tube). The tubes were allowed to incubate overnight.

MBC method

MBC test was used to determine the activity of *S. vagina* AE on *V. harveyi*. The plate agar method (TSA and TCBS) was used for the MBC test. The AE concentrations of 200 mg/L (based on the MIC result) and at least two more concentrated solutions (250 and 300 mg/L) were counted to determine viable CFU/mL. The plates were incubated at 37°C (24-36 h). The MBC value was determined by observing the first clear region on the agar plate (which has no visible bacterial growth) (Parvekar *et al.*, 2020).

Antibacterial assay

This study considered 7 treatments with three replications. The dimensions of the used aquariums were 30×30×50 cm. In the experimental groups, the only variable factor was the concentrations of *S. vagina* AE. Each aquarium contained 6 liters of seawater, which was filtered by a sand filter. A total of 5,000 *L. vannamei* postlarvae were reared in the tanks. After 24 h, AE of S. vagina with concentrations of 100, 150, 200, 250, and 300 mg/L was added to each

[DOR: 20.1001.1.15622916.2022.21.6.5.1]

treatment. Feeding was done twice a day and the tanks were siphoned daily (in the afternoon) during the trial. After 24 h of adding the extract, 10 mL of bacterial inoculation with 5 McFarland turbidity was added to each aquarium. The dead post larvae with typical disease symptoms were collected and analyzed. Histology of the hepatopancreas was performed. The processed tissues were then observed under a microscope. Post-larval biometrics was performed twice (the first day before bacterial infection and the eighth day).

Histology

The collected samples were stored in dark containers comprising Davidson's fixative solution during the test period. The samples with Vibriosis symptoms were isolated from each treatment and placed on the bottom of lace baskets. The samples were very small and therefore, restrained with a net cloth with fine nets in the small baskets were placed in a container of white alcohol to prevent drying. The baskets were removed from the alcohol container after preparing all the samples and placed in a large basket. The large basket was then placed in the tissue-processing apparatus (Histokinette) and adjusted according to Lightner (1996).

The small baskets were removed from the apparatus 12 h later and placed on a heating plate to melt the paraffin around the samples. Molding containers have different sizes that were selected according to the sample size. The samples were placed in a mold and the samples were covered with paraffin by a melting machine. Then the lid of the mold was closed. The molds were then placed on a cooling plate to harden the paraffin inside the mold. The molds were placed in the freezer after cooling. Sections of the molded samples were prepared using a microtome device (with a degree between 3 and 7 microns) and placed on a lam. First, the sections were placed in a cold water bath for a few seconds and then in a hot water bath. They were then removed by lam and placed on a rack to dry. Chrome Alum-Gelatin Adhesive was used to fix the tissue samples and the tissue samples were stained by the hematoxylin-eosin method. The samples were coated with lamel and observed under a microscope (Lightner, 1996).

Results

The MIC of *S. vagina* AE was 200 mg/L (0.25) and the MBC was determined at 250 mg/L (0.1667). The results showed that the extract obtained from *S. vagina* at concentrations of 200, 250, and 300 mg/L could prevent *Vibrio harveyi* bacterial infection in post-larvae of *L. vannamei*. The comparison of post larvae in all groups showed that the mortality rate at the extract concentration of 250 mg/L was significantly reduced. The highest mortality percentages were observed in the extract concentrations of 100 and 150 mg/L, respectively (Fig. 1).

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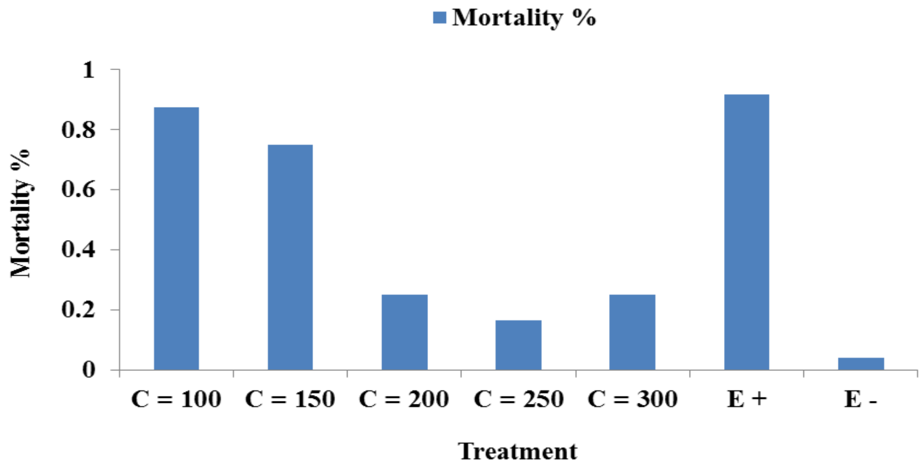

Figure 1: Mortality Percentages of *Litopenaeus vannamei* postlarvae at different concentrations of *Solen vagina* acetone extract (mg/L).

Length growth of postlarvae

The results showed that the extracted acetone from *S. vagina* had a significant effect on the growth of post-larval length at 250 mg/L (p<0.05) but it had no significant effect at the other concentrations of AE. The mean length growth of post-larval at different concentrations of the extract at the end of the eighth day is shown in Figure 2.

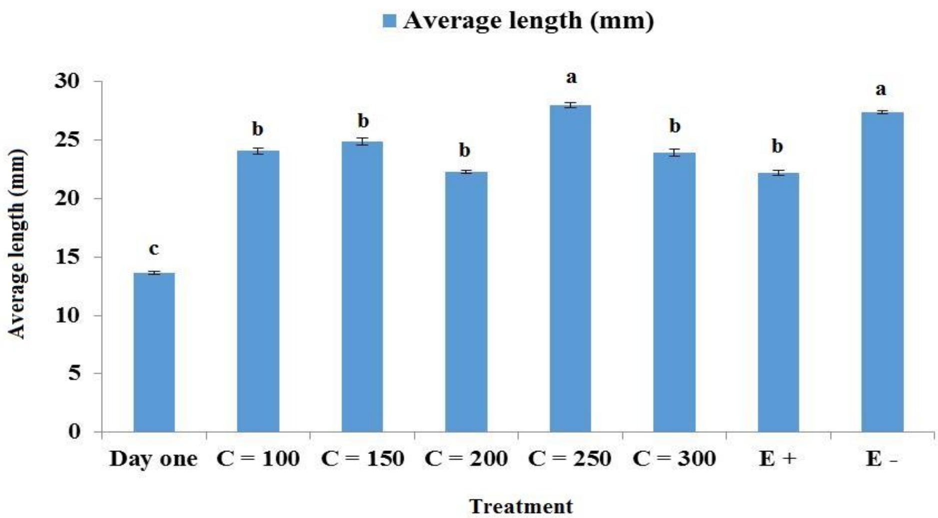

Figure 2: Mean length growth of *Litopenaeus vannamei* postlarvae (mm) at different concentrations (mg/L) of Solen vagina acetone extract at the end of the 8th day. Point a: nearest value to E-, Points b: significant differences with E-, Concentration (mg/L), Length (mm).

Weight growth of postlarvae

The results indicated that the AE of *S. vagina* at 250 mg/L concentration had a significant effect on the weight growth of postlarvae (p>0.05). The mean weight growth of post-larval at different concentrations of the extract at the end of the eighth day is shown in Figure 3.

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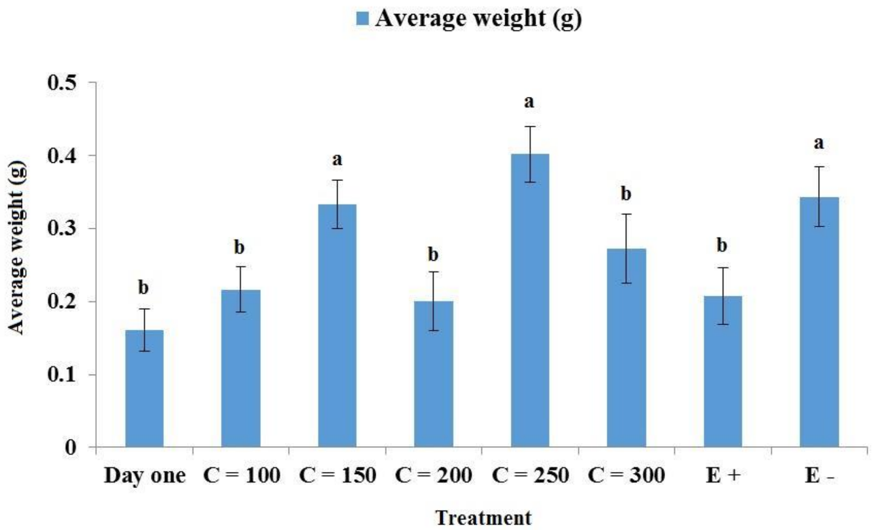

Figure 3: Mean weight growth of *Litopenaeus vannamei* postlarvae (g) at different concentrations (mg/L) of *Solen vagina* acetone extract at the end of the 8th day. Point a: nearest value to E-, Points b: significant differences with E-, Concentration (mg/L), weight (g).

Melanized tissues were observed at concentrations of 100 and 150 mg/L *S. vagina* AE in the hepatopancreas due to Vibrio infection (Figs. 4 and 5). No melanized tissue was observed in the post-larval hepatopancreas at concentrations of 200, 250, and 300 mg/L *S. vagina* acetone extract and all hepatopancreatic cells were normal.

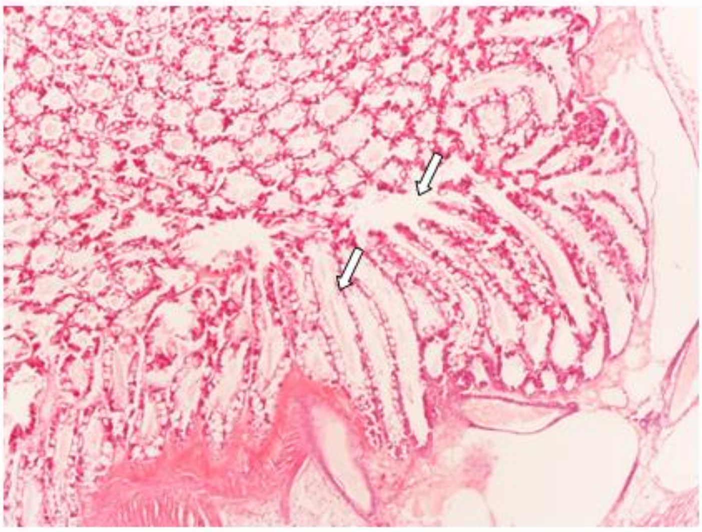

Figure 4: Melanized tissues of hepatopancreas due to *Vibrio Harvey* infection at 100 mg/L concentration of *Solen vagina* acetone extract.

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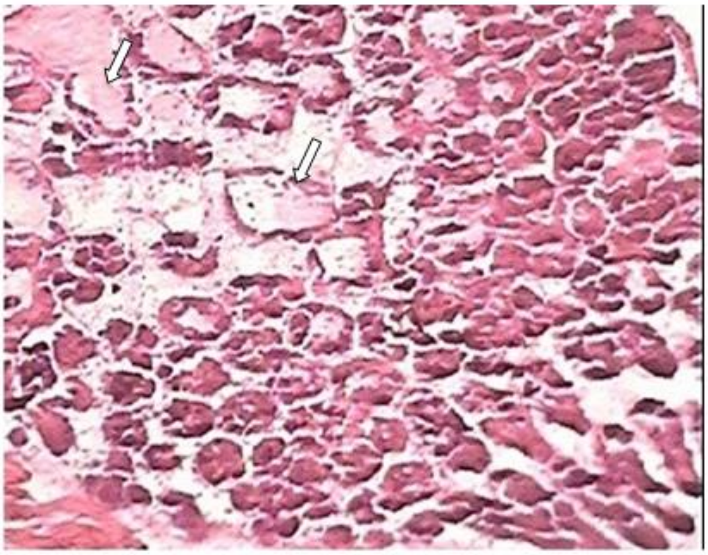

Figure 5: The cellular necrosis of hepatopancreas due to *Vibrio Harvey* infection at 150 mg/L concentration of *Solen vagina* AE (×400).

Discussion

As a filter-feeding organism, mollusks are exposed to different concentrations of pathogens such as bacteria. Various antimicrobial substances have been identified, described, and characterized from mollusks (Hubert *et al.*, 1996; Bullet *et al.*, 2004; Haug *et al.*, 2004; Dorrington *et al.*, 2008). More than a hundred new antimicrobial compounds are isolated from marine invertebrates yearly similar to the bivalves which exhibit a broad spectrum of antimicrobial properties (Bartlet *et al*., 2002). According to the results, *S. vagina* AE with three concentrations of 200, 250, and 300 mg/L could control Vibrios diseases in *L. vannamei* as an antibacterial combination. Among these concentrations, the 250 mg/L had the most effect on the infected post larvae with *Vibrio*, because it was capable to stop *Vibrio harveyi* activity in post-larvae of *L. vannamei*. The lowest level of mortality was observed at a concentration of 250 mg/L which had a significant effect on the length and weight growth of postlarvae. Chellaram *et al.* (2004) considered the antibacterial activity of the oyster *Pteria chinensis* against 10 fish pathogens that the Acetone extract was the most active inhibitor. Wouter *et al.* (2005) investigated the effect of the oyster extract on the yolk production of Kuruma shrimp (*M. Japonicus*) and reported that this extract is a rich source of cholesterol and sex steroids affecting the vitellogenesis process. Huang *et al*. (2006) reported that the use of the ethanolic extract of Sargassum algae at optimal levels of 0.5% and 1.0% for 14 days effectively improved the resistance of *Fenneropenaeus chinensis* shrimp to vibriosis and increased immune activity. Hoa *et al.* (2009) reported the positive effect of oysters on the nutrition of *Penaeus monodon* breeders. Therefore, enrichment techniques and the use of plant and marine animal extracts can be

[DOR: 20.1001.1.15622916.2022.21.6.5.1]

used as food sources to increase disease resistance, increase survival rates, and shrimp production in the aquaculture systems (Adloo *et al*., 2022).

Huynh *et al.* (2011) investigated the immune response of *Litopenaeus vannamei* and its resistance to *Vibrio alginolyticus* and White Spot Syndrome (WSSV) as a result of *Sargassum hemiphyllum* algae extract. The shrimp immersed in the tank containing AE with a concentration of 300 mg/L had greater immune responses and resistance against *V. alginolyticus* infection. Kanjana *et al.* (2011) reported that *Gracilaria fisheri* algae extract could be used to prevent and treat *Vibrio harveyi* in juvenile *P. monodon*. Also, the use of its ethanolic extract has caused a significant increase in the total number of homocytes and granulocytes compared to control shrimp. According to Sirirustananun *et al*. (2011), the survival rate of shrimp (*Litopenaeus vannamei*) fed with food containing algae extract (*Gracilaria tenuistipitata*) was significantly higher than shrimp fed with the control diet against *V. alginolyticus* and WSSV. Therefore, the researchers reported that a diet containing algae extract (*Gracilaria tenuistipitata*) at a rate of 0.1 g/kg could increase the innate immunity of the shrimp within 14 days.

Manilal *et al*. (2012) reported that the use of *Asparagopsis orientalis* red algae extract in the diet could increase the survival rate in the shrimp juveniles infected with *Vibrios*. Kiran *et al.* (2014) considered the antibacterial effect of water and the methanol extracts of the *Perna viridis* and *Nerita albicilla* and revealed that methanol extracts had better antibacterial effects rather than the water extracts. *P. aeroginosa* is extremely inhibited by methanol extracts of *Perna viridis* and *Nerita albicilla* (80% bactericidal activity). Eswar *et al.* (2014) evaluated the antibacterial activity of crude extracts of marine bivalves including *Anadara granosa*, *Placenta pelacenta*, and *Pinctada fucata*. The antibacterial activity was carried out against 10 pathogens such as *V. parahaemolyticus* and *V. cholera*. The results showed that the mollusk extracts can be used as antibacterial agents. Dashtian nasab *et al.* (2016) reported that the ethanolic extract of Persian Gulf algae (*S. angustifolium, L. snyderiae, K. alvarezii,* and *G. corticata*) is beneficial to improve the growth, survival, and control of white legs shrimp (*Litopenaeus vannamei*) diseases in the breeding centers. Karnjana *et al.* (2019) suggested using low concentrations of *Gracilaria fisheri* ethanolic extract in the treatment of *Vibrio parahaemolyticus* and *V. harveyi* in shrimp. Esquer-Miranda *et al.* (2016) reported that the use of methanolic extracts of *Caulerpa sertularioides* and *Ulva lactuca* could be used to prevent *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in shrimp. Huang *et al.* (2020) reported the effects of *Phyllanthus amarus* extract on immune, growth, and resistance responses to *V. alginolyticus* in *L. vannamei* fed. Eidi Ghalehghazi *et al.* (2021) reported the nutritional effect of hot water extract of brown alga *Sargassum ilicifolium* on some hemolymph immunity indices in

L.vannamei is effective to increase antioxidant parameters. No significant changes were observed in the levels of acid phosphatase and phenoloxidase of hemolymph in the shrimp fed with hot algae extract. The shrimp with *P. amarus extract* had a higher survival rate than the shrimp fed with the control diet. Torpee *et al.* (2021) reported that the use of the crude probiotic extract of *Rhodobacter sphaeroides* SS15 in the diet of Vannamei shrimp increased the survival rate of the shrimp exposed to *V. parahaemolyticus.* Therefore, the results of different researchers have shown the positive effect of plant and marine animal extracts to control vibriosis in different shrimps, which is consistent with the results of this study. Because of increasing antimicrobial resistance, there is a need to develop new therapies to prevent the development of resistance and growth of bacteria (Otero-Gonzalez *et al.*, 2010). Marine invertebrates have an effective innate immune system to defend against pathogens. However, antimicrobial peptides of marine invertebrates have not been well-developed. Therefore, the prospect of research is essential to obtain antimicrobial peptides from marine invertebrates to treat bacterial diseases of cultured shrimp (Kiran *et al.*, 2014).

The highest mortality was observed in the groups treated at 100 and 150 mg/L *S. vagina* AE and the lowest value was observed at 250 mg/L. Based on the results, the exposed post larvae showed deformation symptoms in the hepatopancreas cells. Besides, *S. vagina* AE could reduce and prevent mortality caused by *V. harveyi*. MIC of the extract for *V. harveyi* was 200 mg/L. Therefore, *S. vagina* AE can be used as an active ingredient to prevent vibriosis in *L. vannamei*. However, further studies are suggested to investigate other usable extracts of this species with different medicinal and healing properties.

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