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Research Article

Persian Gulf Methanolic extract of *Nerita longii* as Efficient Antioxidant Agent

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Abstract

Background: Marine organisms are the important key for the development and conception of new drugs with medicine applications. Marine invertebrates produce a high amount of natural products as adaptation to their environmental and as result of their life style. Marine natural compounds with powerful bioactivities were already extracted from marine invertebrates including gastropods, tunicates, sponges, soft corals, sea hares, sea cucumbers and bryozoans. Some of those compounds, have pharmaceutical activity such as antitumor, antimicrobial, anti-inflammatory and antioxidant activities which can be used in clinical and preclinical assays.

Objectives: The purpose of this research was to examine the activity of methanolic extract from Persian Gulf gastropod, *Neritalongii* as efficient antioxidant.

Materials and methods: Persian Gulf gastropod, *Neritalongii* was washed and macerated with ethyl acetate and methanol for three days. The resulting extracts were dry with freeze dryer and the final residue were consider for antioxidant tests by DPPH, FRAP and FOLIN methods.

Results: In DPPH method, the resultant IC50 for methanol and ethyl acetate were 8.07 and 10.13 mg/ml, respectively. In FRAP method, the resultant EC1 for methanol and ethyl acetate were 3.687, 5.35 mg/ml respectively. In FOLIN method, the highest Absorbance in methanolic and ethyl acetate extracts is given 1.626 and 1.592.

Conclusions: Muscle tissue of Persian Gulf gastropod, *Neritalongi*, is an important source of natural proteins and peptides. So high antioxidant activity was given from FRAP, DPPH and FOLIN methods of methanolic extract can be related to the ability of methanol to extract corresponding peptides and proteins.

Introduction

The Ocean covers around 70% of the Earth surface. Humans especially depend on marine systems for a high number of their practices such as food resources, ways to travel around, business and more recently as a source of important Metabolites for the cosmetic and pharmaceutical industries. In the last decades the high bioactivity studies of compounds found in marine organisms have turned sea life into a new and prolific source of metabolites, which can be very efficient to improve human health and life quality. Those compounds present a wide range of biological activities

such as anti-tumor, anti-microbial, anti-inflammatory and anti-oxidant. Therefore, marine organisms are considered to be the key for the development and conception of new drugs with medicine applications [1].

Marine invertebrates produce a high amount of natural products as adaptation to their environmental and as result of their life style. As a result of living in aquatic system, those organisms have face specific biochemical and physiological constrains such as darkness, predation, exposure to ultra-violet radiation, lack of physical defense (soft body), cold temperatures and high pressurized environment's [2].

Around 10000 marine natural compounds with powerful bioactivities were already extracted from marine invertebrates including gastropods, tunicates, sponges, soft corals, sea hares, sea cucumbers and bryozoans. Some of those compounds, have pharmaceutical activity such as antitumor, antimicrobial, anti-inflammatory and antioxidant activities which can be used in clinical and preclinical assays, leading to the 4 development of new medicines as the example of Ziconitide (Proalt TM), used to treat chronic pains, which was obtained from a mollusk specie, *Conus magus* [1,3-5].

In our body, oxidation process leads to cell damage, cancer and degenerative diseases; antioxidant molecules present in different mollusks prevent cell damage from oxidation reaction [6]. Compounds isolated from molluscs were also used in the treatment of rheumatoid arthritis and osteoarthritis [7]. Mollus can extracts also exhibited antiviral and antibacterial activity against fish pathogenic bacteria, and the extract also may be applied in aquaculture [8].

In general very fewer studies have been done on the antioxidant activity of gastropods while some of them have been estimated as whole body homogenate which possesses a range of antimicrobial compounds. The gastropod egg and tissue consists of active secondary metabolites that have more antimicrobial activities [9].The antioxidant compounds are essential to trap free radicles and reduce the risk of cancer and heart disorders [10-12]. The large number of works has been done in other organisms, but only a few researches were done on mollusks [13]. The resources of gastropods are not well utilized in the Persian Gulf region. The objective of the present study was to investigate the antioxidant activity of the body tissues of Nerita Longii sp. gastropod around the Persian Gulf region, Bushehr coasts of Iran.

Materials and Methods

Chemicals

2,2-DiPhenyl-2-Picrylhydrazyl (DPPH), 2,4,6-TriPyridyl-s-TriaZine (TPTZ) and ferulic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Folin-Ciocalteu reagent, ferric chloride ($FeCl_3$.$6H_2O$), and methanol and ethyl acetate were from Merck (Darmstadt, Germany)

Sample collection and extraction

Live specimens of *Nerita longii* gastropod was collected from the Bushehr coast of Persian Gulf of Iran and directly brought to the laboratory and identified by scientists from Marine Science and Technology of Khoramshahr, Khuzestan, Iran. The whole body muscle was taken out from the shell, used for extraction by methanol and stored for 4 days.

Preparation of extracts

The shells were separated in lab and the whole muscle tissue weighing 80 g was macerated with ethyl acetate and methanol for three days. The supernatant solvent was concentrated using vacuum evaporator (35-55oC) under reduced pressure and the resultant extract was dry with freeze dryer and kept in clean glass vials at -80 oC until use.

DPPH radical scavenging assay

The free radical scavenging activity of methanol and ethyl acetate extract of *N. Longi* was assessed using DPPH (1,1-diphenyl-2-picryl hydrazyl) radical [14]. Due to the poor solubility of methanol and ethyl acetate extract in aqueous medium it was dissolved in 1% DMSO and later on made up to desired concentrations with distilled water. The extract in different concentrations were added to 0.1 ml of 1 M Tris-HCl (pH 7.9), and then mixed with 0.6 ml of DPPH (100 µM) in methanol for 20 min at room temperature under protection from light. Absorbance of the mixture was read on a UV-VIS spectrophotometer (Hitachi, U-2001) at 517nm against the blank. Butyrate Hydroxyl Toluene (BHT) was used as the standard antioxidant and the percentage inhibition of test and the standard were calculated as % Inhibition = (($A_{Control}$-A Test) / $A_{Control}$) x 100

Where $A_{Control}$ is the absorbance of the control (without extract) and A Test is the absorbance of the sample of extract and standard.

Ferric Ion Reducing Antioxidant Power Assay

The FRAP assay also takes advantage of electron-transfer reactions. Herein a ferric salt, Fe (III) $(TPTZ)_2 Cl_3$ (TPTZ) 2, 4, 6-tripyridyls-triazine), is used as an oxidant [15-17]. The FRAP assay involves the following procedures: The oxidant in the FRAP assay is prepared by mixing TPTZ (2.5 mL, 10 mM in 40 mMHCl), 25 mL of acetate buffer, and 2.5 mL of $FeCl_3$âH$_2$O (20 mM). The conglomerate is referred to as "FRAP reagent". The final solution has Fe (III) of 1.67 mM and TPTZ of 0.83 mM. Therefore, the

TPTZ is deficient as the ideal reaction stoichiometry between Fe (III) and TPTZ is 1 to 2. The oxidant is not just Fe (III)(TPTZ)2, it also contains other Fe(III) species which can lead to potential problems as many metal chelators in food extract could bind Fe (III) and form complexes that are also capable of reacting with antioxidants. To measure FRAP value, 300 µL of freshly prepared FRAP reagent is warmed to 37 °C and a reagent blank reading is taken at 593 nm; then 10 µL of sample and 30 µL of water are added.

Absorbance readings are taken after 0.5 s and every 15 s until 4 min. The change of absorbance (¢A) A4min - A0min) is calculated and related to ¢A of a Fe (II) standard solution. ¢A is linearly proportional to the concentration of antioxidant. One FRAP unit is arbitrarily defined as the reduction of 1 mol of Fe (III) to Fe (II). The FRAP values for ascorbic acid, R-tocopherol, and uric acid are identical (2.0). The FRAP value of bilirubin is 1-fold higher than that of ascorbic acid. These results suggest that 1 mol of vitamin C can reduce 2 mol of Fe (III) and that 1 mol of bilirubin can reduce 4 mol of Fe (III). This is in conflict with the fact that both vitamin C and bilirubin are two-electron reductants. It is known that when bilirubin is oxidized, it is transformed to beliverdin (by losing two hydrogenatoms, not just electrons), which happens to have an absorption at 593 nm with coeffiicency (593) 1-104) comparable with that of Fe (II) (TPTZ) 2.50Pulido and co-workers (51) measured the FRAP values of several polyphenols in water and methanol. However, the absorption (A593) does not stop at 4 min; instead, it slowly increased even after several hours. Polyphenols with such behaviors include caffeic acid, tannic acid, ferulic acid, ascorbic acid, and quercetin. The FRAP values of these compounds cannot be obtained accurately if 4 min reaction time was followed.

Total Phenols Assay by Folin-Ciocalteu Reagent

FCR was initially intended for the analysis of proteins taking advantage of the reagent's activity toward protein tyrosine (containing a phenol group) residue [18]. Many years later, Singleton and co-workers extended this assay to the analysis of total phenols in wine; since then the assay has found many applications. The FCR-based assay gained popularity and is commonly known as the total phenols (or phenolic)

Assay: The FCR actually measures a sample's reducing capacity, but this is not reflected in the name "total phenolic assay". Numerous publications applied the total phenols assay by FCR and an ET-based antioxidant capacity assay (e.g., FRAP, TEAC, etc.) And often found excellent linear correlations between the "total phenolic profiles" and "the antioxidant activity". This is not surprising if one considers the similarity of chemistry between the two assays. One of the assays may just be redundant. A recent report of using polyphenol oxidase for assaying total phenols in tea may be more specific to phenolic compounds.

The FCR is typically made by first boiling (for 10 h) the mixture of sodium tungstate (Na_2WO_4, $2H_2O$, and 100 g), sodiummolybdate (Na_2MoO_4, $2H_2O$, 25 g), concentrated hydrochloric acid (100 mL), 85% phosphoric acid (50 mL), and water (700 mL). After boiling, lithium sulfate (Li_2SO_4, $4H_2O$, 150 g) is added to the mixture to give intense yellow solutions the FC reagent. Contamination of reductants leads to a green color, and the addition of oxidants such as bromine can restore the desired yellow color. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates-molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly (PMoW11O40)4-. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI): Mo (VI) + e → Mo (V).

Obviously, the FC reagent is nonspecific to phenolic compounds as it can be reduced by many non-phenolic compounds [e.g., vitamin C, Cu (I), etc.]. Phenolic compounds react with FCR only under basic conditions (adjusted by a sodium carbonate solution to pH-10). Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing FCR. This supports the notion that the reaction occurs through electron transfer mechanism. The blue compounds formed between phenolate and FCR are independent of the structure of phenolic compounds, therefore ruling out the possibility of coordination complexes formed between the metal center and the phenolic compounds.

Despite the undefined chemical nature of FCR, the total phenols assay by FCR is convenient, simple, and reproducible.

As a result, a large body of data has been accumulated, and it has become a routine assay in studying phenolic antioxidants.

Statistical analysis

Statistical analysis was done using SPSS version 14. IC50 values respectively for DPPH were found out by Linear Regression Probit analysis.

Results and Discussion

DPPH radical scavenging activity

DPPH radical scavenging property of *N. Longi* is shown in Figure 1. The methanolic extract showed a dose dependent pattern in DPPH radical scavenging in the range of concentrations tested (0.312-20 mg/ml). The IC50 value of the methanolic extract was found to be 8.07 mg/ml. The higher concentration of methanol extract showed a percentage inhibition of 82.86 %.

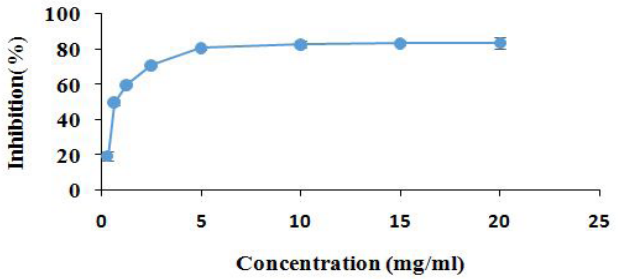

Figure 1: Effect of methanol extract of *N. Longion* DPPH radical scavenging activity.

As showed in Figure 2, the EtOAc extract have a dose dependent pattern in DPPH radical scavenging in the range of their concentrations (0.312-20 mg/ml). The IC50 value of the EtOAc extract was found to be 10.13 mg/ml. The higher concentration of EtOAc extract showed a percentage inhibition of 76.04 %.

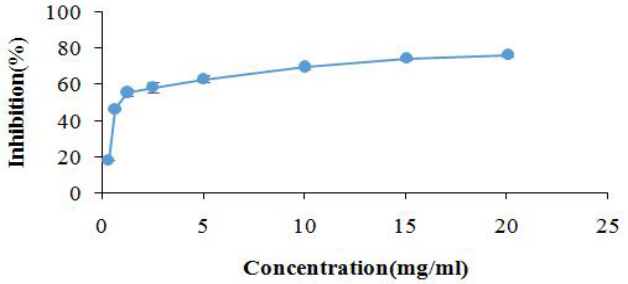

Figure 2: Effect of EtOAc extract of N. Longi on DPPH radical scavenging activity.

As showed in Figure 3, the resultant IC50 for methanol and ethyl acetate were 8.07 and 10.13 mg/ml, respectively.

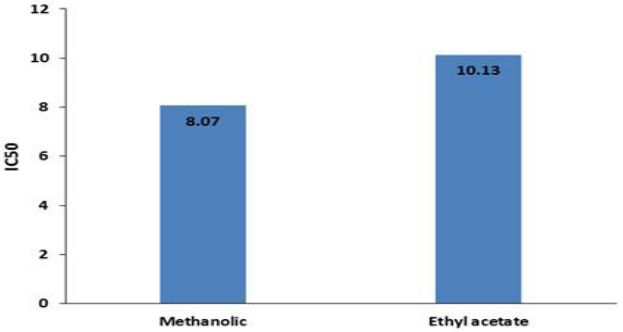

Figure 3: Effect of IC50 on Methanol and EtOAc extract of N. Longi on DPPH radical scavenging activity

Ferric reducing activity based on FRAP assay

The ethanolic extract of Sulawesian beans exhibited the highest antioxidant potential among the extracts (Figur 6), based on the FRAP assay. FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe3+-TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe2+-TPTZ) [15]. Generally, the reducing properties are associated with the presence of compounds, which exert their action by breaking the free radical chain through donating a hydrogen atom [19,20]. According to [15], the reduction of Fe3+-TPTZ complex to blue colored of Fe2+-TPTZ occurs at low pH.

Sulawesi an and Malaysian beans are well known to have a low cotyledon pH, while Ghanaian has medium pH, and Ivory Coast has high pH [21]. The highest antioxidant potential of Malaysian beans could be due to the highly acidic (low pH) nature of the bean cotyledon, which many influence the pH of the assay medium.

As depicted in the Figure 4, the methanolic extract showed a dose dependent pattern in FRAP scavenging in the range of their concentrations (0.312-10 mg/ml). The EC1 value of the methanolic extract was found to be 3.687 mg/ml. The higher Absorbance of methanolic extract showed at1.78.

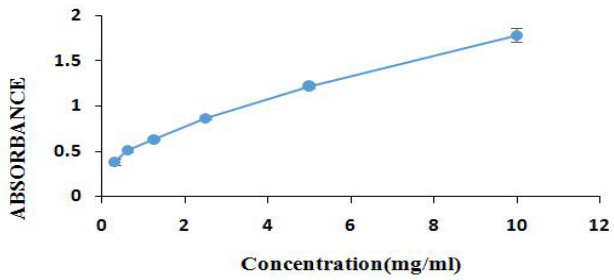

Figure 4: Effect of methanolic extract on Absorbance of N. Longi in FRAP Value

As depicted in Figure 5, the EtOAc extract showed a dose dependent pattern in DPPH radical scavenging in the range of concentrations tested (0.312 -20 mg/ml). The EC1 value of the EtOAc extract was found to be 5.35 mg/ml. The higher concentration of EtOAc extract showed the absorbance at1.822.

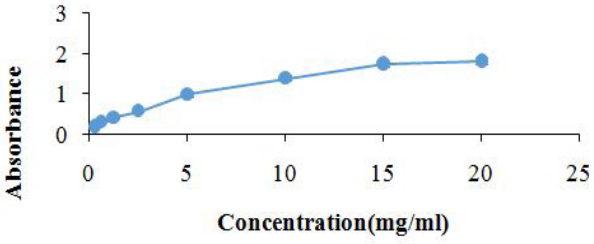

Figure 5: Effect of EtoAc extract on Absorbance of N. Longi in FRAP Value

As shown in Figure 6, the resultant EC1 for methanol and ethyl acetate were 3.687, 5.35 mg/ml respectively.

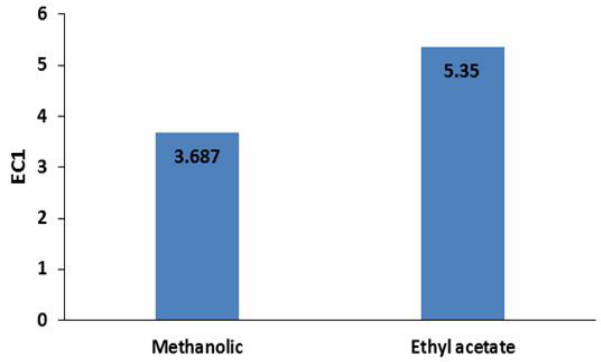

Figure 6: The comparison of EC1 in methanolic and EtOAc extract of FRAP Value

Total phenolic content

Several studies showed a correlation between antioxidant activity and phenolic content [22-24]. As shown in Figure 7, methanolic extract beans had the highest phenolic content. The highest Absorbance in methanolic extract is 1.626.

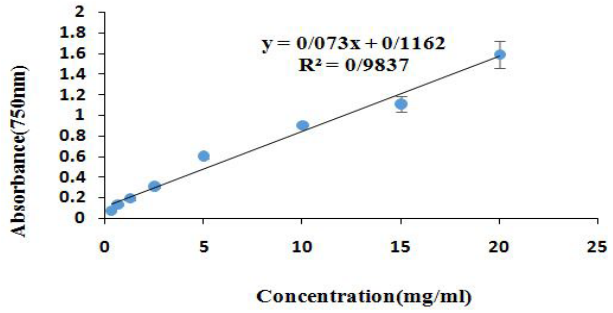

Figure 7: Effect of methanolic extract on Absorbance of *N. Longi* in FOLIN

As shown in Figure 8 the highest Absorbance in EtOAc is 1.592.

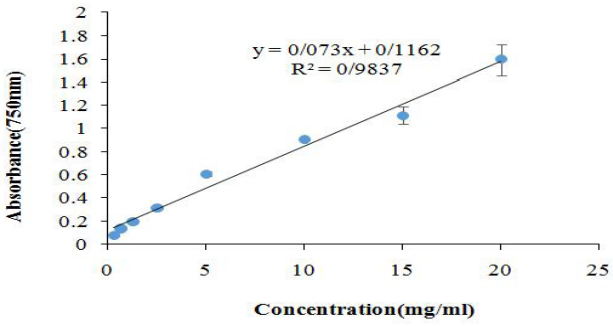

Figure 8: Effect of EtOAc extract on Absorbance of N. Longi in FOLIN

Conclusion

Muscle tissue of Persian Gulf gastropod, Nerita Longi, is an important source of natural proteins and peptides. So high antioxidant activity was given from FRAP, DPPH and FOLIN methods of methanolic extract can be related to the ability of methanol to extract corresponding peptides and proteins.

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