COMU J Mar Sci Fish 1(2): 66-71
e-ISSN 2651-5326

Journal Home-Page:
http://jmsf.dergi.comu.edu.tr
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COMU JOURNAL OF MARINE SCIENCE AND FISHERIES

Research Article

Antioxidant and Antimicrobial Activity of Sea Cucumber (*Holothuria tubulosa*, Gmelin 1791) Extracts

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(Received: 21.11.2018; Accepted: 20.12.2018)

Abstract: *Holothuria tubulosa* is an economic and most harvested sea cucumber species found in Turkey seas. Sea cucumbers are known to be sources of some of bioactive components which are play important roles in human health. In this study, it was aimed to determine the antioxidant and antimicrobial activity of *H. tubulosa*. The extracts of *H. tubulosa* were obtained with using different solvents such as, acetonitrile/trifloroacetic acid, methanol, and water/methanol. According to results, antioxidant activity of the extracts was lower than the reference antioxidant agents at all concentrations. However, the activity at higher concentrations of extracts was found to be closer to reference agents. Bacteriostatic effect was observed after the concentration of 12 µg/ml for all bacteria except *Bacillus cereus,* which has also shown resistance to reference antibiotic. As a consequence, *H. tubulosa* found in Turkey seas may be regarded as potential source of natural antioxidant agent.

Keywords: *Holothuria tubulosa*, Sea Cucumber Extract, Antimicrobial Activity, Antioxidant Activity, DPPH.

Deniz Hıyarı (*Holothuria tubulosa*, Gmelin 1791) Ekstraktlarının Antioksidan ve Antimikrobiyal Aktivitesi

Özet: *Holothuria tubulosa* Türkiye denizlerinde bulunan ekonomik ve en çok hasat edilen deniz hıyarı türlerindendir. Deniz hıyarları insan sağlığı için önemli olan bioaktif bileşiklerin kaynağı olarak bilinmektedir. Bu çalışmada *H. tubulosa*'nın antioksidan ve antimikrobiyal etkisinin belirlenmesi amaçlanmıştır. *H. tubulosa* ekstraktları, asitontril/trifloroasetik asit, methanol ve su/metanol gibi farklı çözücüler kullanılarak elde edilmiştir. Sonuçlara göre, ekstraktların antioksidan aktiviteleri, referans antioksidanlara göre tüm konsantrasyonlarda daha düşük belirlenmiştir. Ancak, yüksek konsantrasyonlarda, ekstraktların antioksidan aktivitesi referans antioksidanlara yakın derecede bulunmuştur. Bakteriyostatik etki 12 µg/ml konsantrasyonundan sonra belirlenmiş, ancak bu etki hiçbir konsantrasyon ve ekstrakta, *Bacillus cereus* için gözlemlenmemiştir. Sonuç olarak, Türkiye denizlerinde bulunan *H. tubulosa*'nın, potansiyel doğal antioksidan kaynağı olabileceği düşünülmektedir.

Anahtar Kelimeler: *Holothuria tubulosa,* Deniz Hıyarı Ekstraktı, Antimikrobiyal Aktivite, Antioksidan Aktivite, DPPH.

Introduction

Sea cucumbers belonging to the Holothuroidea class are a different group of worm-like and soft-bodied echinoderms, which live nearly all marine environment. Sea cucumbers are represented by approximately 1600 species worldwide and 66 species of these species are commercially exploited (Purcell, 2010). *Holothuria tubulosa* is a sea cucumber species, which is one of the most harvested and economic echinoderms found in Turkey Seas (Çaklı et al., 2004; Aydın, 2008; Aydın et al., 2011). Natural beds of this species can be found at Northern Aegean to Black Sea in Turkey (Öztoprak et al., 2014). Global trade, consumption, and utilization of sea cucumbers as fresh, frozen or bech-de-mer, a processing technique which is constituted by salting and drying processes, mostly occur around Asian countries. However, they are also considered as valuable seafood by most of other countries, even if not there is consumption. The reason of being valuable of sea cucumbers is not just involved with their nutritional values, but also with their therapeutic and pharmaceutical properties. These properties are mainly shaped with the biochemical composition of sea cucumber. Recent studies have shown that some sea cucumber species have important components in their soluble extracts, which are antioxidative and antimicrobial characteristics

(Nobsathian et al., 2017; Mashjoor et al., 2018). Thus, last decades, most researchers have tried to determine the quality and importance of their endemic sea cucumber species. To our knowledge, there is limited study on *H. tubulosa* about this subject in literature. For this reason, the aim of the study was to determine some of bioactive properties such as antioxidant and antimicrobial activity of the soluble extracts of *H. tubulosa*, which is economically important and one of the most harvested sea cucumber species found in Turkey Seas.

Material and Methods

Total of 120 *H. tubulosa* species were collected by scuba diving up to 15 meters depth from the Southern Coasts of Çanakkale Strait in Marmara Sea, Turkey. Collected samples were transported to laboratory at 4ºC ± 2 via a cool box in 2 hours. Mean total length and total weights of samples were 14.93±0.32 cm and 114.23±4.92 g respectively. After live specimens reached to laboratory, their body wall were cut from abdomen and cleaned from viscera. Remained body walls of samples were used directly in analysis.

Solvent Extraction of Samples

Samples were cut into small pieces (<1 cm^2) and leave to oven (Nüve, FN 500) at 70°C for 20 hours for drying. Dried samples grinded into powder and mixed with water, water + methanol, and acetonitrile at 1:10 (v/w) + % 0.1 trifloro acetic acid (6:4, v/v). The suspensions were mixed continuously at 4°C ± 1 for 24 hours. At the end of continuous stirring, samples were centrifuged 6000 x g for 20 min. The supernatant of samples after centrifugation were collected and the solvents in supernatants were lyophilized (Bluewave, BW-10) and stored at -20ºC ± 2 till used (Esmat et al., 2013).

Antioxidant Activity of Extracts

For DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity, the method described by Sun et al. (2009) was used with slight modifications. Briefly, lyophilized samples were rehydrated with distilled water and serially diluted 50 to 0.50 mg/ml. 1 ml of diluted extract samples were mixed with 4 ml 0.004 % of DPPH-methanol solution. After incubation at 25 ºC ± 0.5 (Nüve, EN 500) for 30 min in dark, the mixtures were centrifuged (Nüve, NF 800 R) at 4000 x g for 10 min. The supernatants of centrifuged samples were placed into spectrophotometer (Optizen 3220UV) and read their absorbances at 540 nm. As control, 1 ml Tris-HCl buffer + 4 ml 0.004 DPPH-methanol was measured at same concentrations. DPPH scavenging capacities of samples were determined according to the formula described below;

$$E\ \% = [(A_{control} - A_{sample})/A_{control} \times 100]$$

$A_{control}$: Absorbance value of the control sample

A_{sample}: Absorbance value of the samples

Iron chelating activity tests were performed according to the method described by Aleman et al. (2011). 1 ml of diluted extract samples placed into tubes which contained 2.7 ml ethanol, 0.1 ml $FeCl_2$ and 0.2 ml ferrozine. After vortexing (LLG, Unitexer), the tubes were incubated at 25ºC ± 1 for 10 min in dark. After incubation, samples were filtered from a basic filter paper and their absorbances were measured at 562 nm. As control, 1 ml of buffer + 3.7 ml ethanol and 0.1 ml $FeCl_2$+0.2 ml ferrozine mixture was used. Chelating activity was calculated according to following equation;

$$DSA\ \% = [\ 1- (A_{sample} / A_{control})] \times 100$$

$A_{control}$: Absorbance value of control sample

A_{sample}: Absorbance value of samples

Reducing power capacities of samples were determined with using the method of Liu et al. (2012). 0.5 ml of diluted sample was mixed with 2.5 ml 0.2 mol/l phosphate buffer (pH 6.8) and 2.5 ml 1% of potassium ferricyanide. The mixtures were incubated at 50ºC ± 1 for 20 min, and 2.5 ml of 10% trichloric acetic acid was added. The mixtures were centrifuged at 8000 x g for 10 min. 2.5 ml of supernatants of centrifuged samples were taken and mixtured with 2.5 ml distilled water and 1 ml 0.1 % $FeCl_3$. Water replaced diluted samples and used as control. The absorbance values of samples at 700 nm were measured and the higher absorbance values recorded as higher reducing power capacity. All antioxidant activity tests were compared with vitamin C, butylated hydroxytoluene (BHT), and ethylene diamine tetra acetic acid (EDTA) as reference antioxidants and chelating agent.

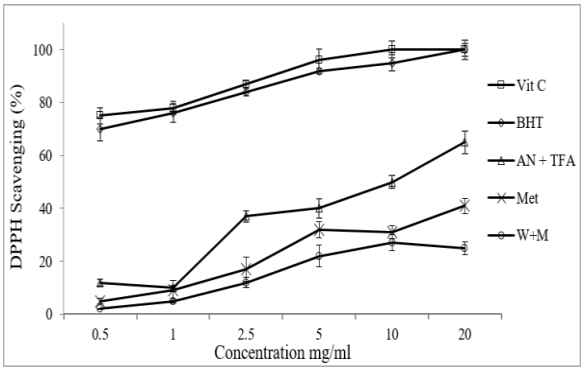
For antimicrobial activity tests, *Salmonella typhimurium* (ATCC 51812), *Bacillus cereus* (ATCC 7464), *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923) bacterial strains were used. Each bacteria were suspended at Muller Hinton Broth (MHB) and incubated (Nüve, ES 120) overnight prior use. The level of bacteria found in inoculums was adjusted in MHB with using a McFarland turbidity device. 96-well plate was used for tests. The starting concentrations of extracts and reference antibiotic (amoxicillin/clavulanic acid) were 50 µl/ml and serially diluted using ½ of prior concentration for followed wells. 100 µl of bacterial suspensions added to all extracts added wells, and the plates were incubated at 35ºC ± 0.5 for 18 hours. The absorbance values of 96-well plates at 600 and 630 nm were measured with using an Elisa reader (Thermo Multiscan 80) (Devienne & Raddi, 2002).

The data were expressed as mean ± standard deviation (SD). Difference of each antioxidant activity and antimicrobial activity of groups was analyzed from the variance (one-way ANOVA). Differences at a 95% ($P < 0.05$) confidence level were considered

statistically significant. All analyses were carried out in triplicate.

Results and Discussion

In this study, *H. tubulosa* extracts were obtained with different solvents and their antioxidant and antimicrobial activities have been determined. Antioxidant capacity of substances can be measured with wide variety tests. Among these test, DPPH radical scavenging activity is a commonly used parameter to determine the potential of antioxidant compounds. DPPH is a stable radical with a maximum absorbance in ethanol at 517 nm (Molyneux, 2004). When DPPH is swept away from an antioxidant compound by taking an electron or a proton, the radical turns its own color to yellow and the absorbance decreases (Peng et al., 2009). DPPH scavenging activities of *H. tubulosa* extracts were summarized in Figure 1. At the starting concentration of this test, 0.5 mg/ml, vitamin C and BHT have shown about 70% scavenging capacity while, extract samples limited around at 10 %. At the 20 mg/ml concentration reference samples have shown 100% activity and extracts have shown 25 to 65 percent of DPPH radical scavenging capacity.

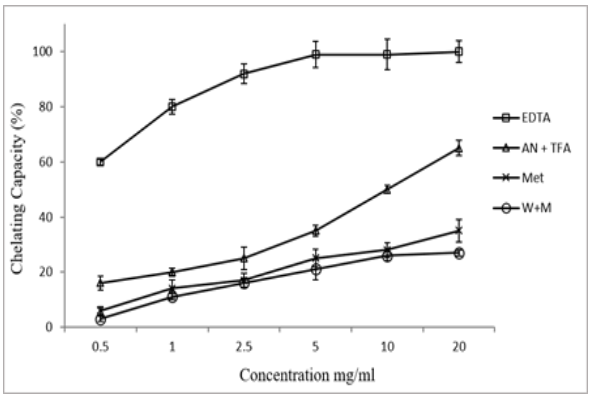


*AN+TFA: acetonitrile + trifloroacetic acid, Met: methanol, W+M: water + methanol

Figure 1. DPPH radical scavenging activity of *H. tubulosa* extracts in comparison with Vitamin C and Butylated hydroxytoluene (BHT).

In this study, maximum 65 percent of DPPH scavenging activity was determined, while Vitamin C and BHT have shown 100 percent at the same concentration (20 mg/ml). The extract obtained from acetonitrile-trifloroacetic acid has shown similarity with methanolic extract of *Holothuria atra* which has 75 % DPPH scavenging capacity (Nobsathian et al., 2017). On the other hand, water composed extraction has lower DPPH scavenging capacity than just methanol extract and this finding was found to be opposite the results reported by Althunibat et al. (2013). The researchers have reported that water extraction of sea cucumbers, namely *Holothuria edulis* and *Stichopus horrens* has higher antioxidant activity than the extracts obtained with methanol (Althunibat et al., 2013). In another study, Murniasih et al. (2015) have reported that the DPPH scavenging activity of *H. atra*, *H. scabra*, *H. excellens*, and *H. leucospilota* at low concentrations (< 1mg/ml) were under 10 %, while ascorbic acid, their reference antioxidant, was reported higher than 50 %. Authors have also concluded that *H. leucospilata* and *H. atra* have antioxidant potentials due to their phytochemical constituents such as, flavanoid, phenols, saponins, and glycoside (Murniasih et al., 2015). Similarly, in our study, the extracts lower than the 1 % concentration have shown 1 to 16 % percent DPPH radical scavenging activity.

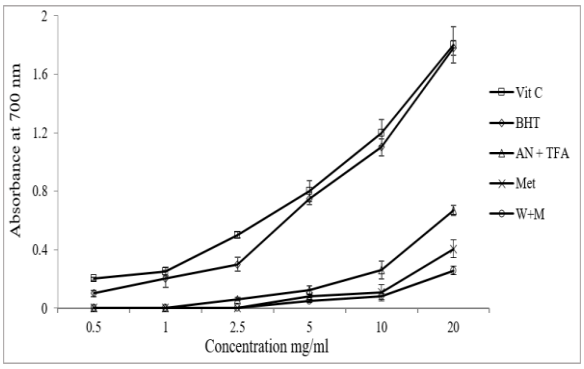
Trace elements ions like iron (Fe^{+2}), catalyze the formation of reactive oxygen species such as hydroxyl radical and superoxide anion. In particular, Fe^{+2} decomposes lipid hydroperoxides, an important reactive oxygen species, during the fenton reaction. Potentially these free radicals accelerate the formation of some oxidative stress-related diseases. Therefore, these metal ions are inactivated by chelating with anti-oxidative agents and the oxidation reactions are delayed (Baugatef et al., 2012). Iron chelating activity of *H. tubulosa* extracts was measured with the inhibition of Fe^{+2} – ferrozine complex formation. EDTA was used as reference chelating agent and results were given in Figure 2. EDTA has shown 60% inhibition of Fe^{+2} – ferrozine formation at the concentration of 0.5 mg/ml, while *H. tubulosa* extracts stayed under 10% at the same concentration. After 5.0 mg/ml EDTA has shown 100% inhibition, however extracts reached maximum (60%) inhibition at 20 mg/ml concentration.



*AN+TFA: acetonitrile + trifloroacetic acid, Met: methanol, W+M: water + methanol

Figure 2. Iron chelating capacity of *H. tubulosa* extracts in comparin with Ethylene Diamine Tetra Acetic Acid (EDTA).

Similar to DPPH scavenging activity, iron chelating activity was found higher for acetonitrile/trifloroacetic acid than other extracts. Esmat et al. (2013) have reported that the sea cucumber extracts can show up to 57 % Fe^{+2} chelating activity even in lower concentrations (150 to 600 µg/ml). These reported results by Esmat et al. (2013) is relatively higher than our results which are lower than 20 percent of Fe^{+2} chelating capacity at 500 µg/ml. It is thought that this difference is originated first from the species differentation and followed by the handling and extraction procedures.



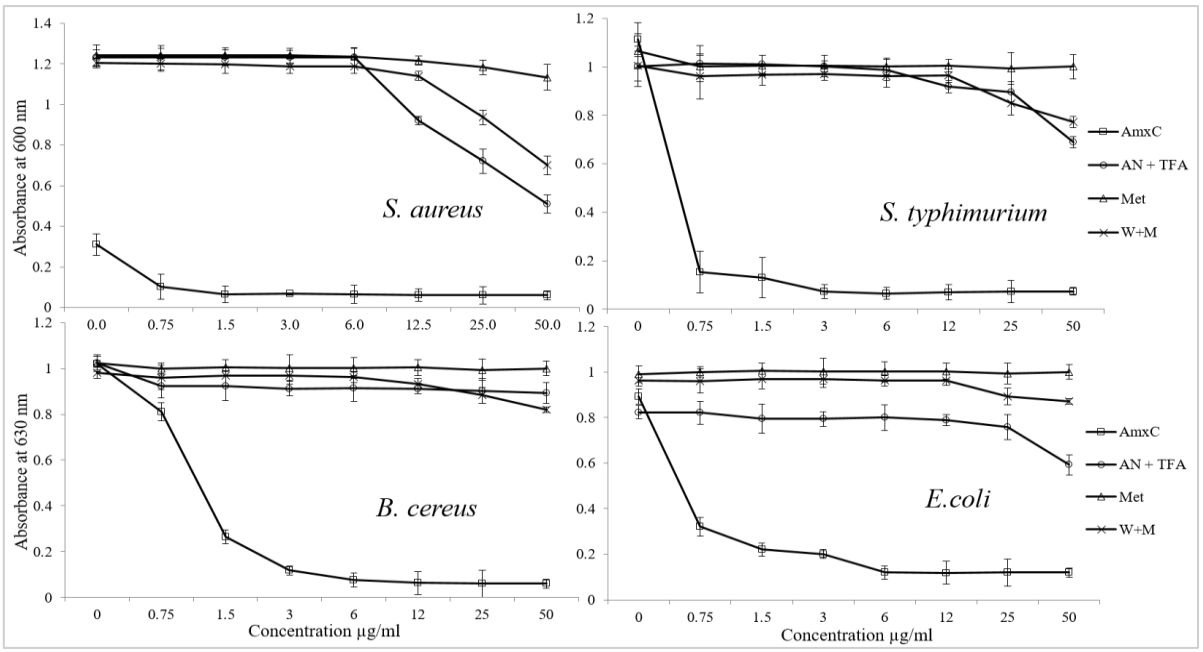
*AN+TFA: acetonitrile + trifloroacetic acid, Met: methanol, W+M: water + methanol

Figure 3. Iron-ferricyanide complex reducing capacity of *H. tubulosa* extracts in comparison with Vitamin C and Butylated hydroxytoluene (BHT).

differences between reference agents and extracts dramatically increased. At maximum concentration (20 mg/ml), extracts of *H. tubulosa* were nearly 1:3 of reference agents.

Reducing capacity of *H. tubulosa* extacts were found lower impact than DPPH and chelating capacity tests. However, reducing capacity results have shown similarity with the values reported by Mashjoor et al. (2018), whose report the iron reducing power absorbance value of methanolic and ethyl extracts of *Holothuria leucospilota*, *Holothuria parva*, and *Holothuria scabra* in the range of 0.33 to 0.66 nm.

Antimicrobial activity of *H. tubulosa* extract on *Salmonella typhimurium*, *E. coli*, *Bacillus cereus,* and *Straphylococcus aureus* is summarized in Figure 4. Antimicrobial activity of the extract was compared



*AmxC: amoxicillin/clavulanic acid, AN+TFA: Aceto Nitrile + Trifloroacetic acid, Met: methanol, W+M: water + methanol.

Figure 4. Antimicrobial activity and minimum inhibation concentrations of *H. tubulosa* extracts against *S. aureus*, S. *typhimurium*, *B. cereus*, and *E. coli* in comparison with amoxicillin/clavulanic acid.

Reducing power capability is related to molecules stabilizing free radicals that are unstable in the environment by giving electrons or hydrogen donors. Molecules with high reducing-power capacity terminate radical chain reactions quickly and have a higher absorbance value at a specific wavelength in the spectrophotometer (Qi et al., 2016). Along with the antioxidant activity of the reducing power, the antioxidants in the reducing power convert the Fe^{+3} / ferricyanide complex into iron form (Bougatef et al., 2012). Reducing powers of *H. tubulosa* extracts and reference agents were summarized in Figure 3. Vitamin C and BHT have shown similarity at same concentrations while *H. tubulosa* extracts were lower than both of these. After 2.5 mg/ml concentrations

with amoxicillin-clavulanic acid, which is wide range spectrum antibiotic and it is used for medinical treatments widely.

The minimal inhibation concentration of extracts on *Salmonella typhimurium*, *Staphylococcus aureus*, and *Escherichia coli*, were determined as 50.0, 12.5, and 50.0 mg/ml, respectively. *Bacillus cereus* has shown resistance to *H. tubulosa* extracts at all concentrations. Antimicrobial activity of *H. tubulosa* extracts was found lower than amoxicillin/clavulanic acid in all concentrations. The minimal inhibation concentration of extracts were observed at 12.5 and 24 µg/ml for tested organisms except for *B. cereus.* In general, obtained results for antimicrobial activity of *H. tubulosa* extracts can be considered as bacteriostatic status rather than bactericidal effect, in the range of tested concentrations. Similar results which highlight the low bactericidal effect or only bacteriostatic effect of sea cucumbers extracts have been reported by reasearchers. Dobretsov et al. (2009) have reported that water and methanol extracts of *H.*

atra and *H. edulisa* were not able to show any inhibition effect on tested bacteria. However, they also reported that bacteriostatic effect can be seen in water extracts of both sea cucumber species (Dobretsov et al., 2009). On the other hand, Mashjoor et al. (2018) have reported that the methanolic extracts of some sea cucumbers and their organs have higher inbitory effect than reference antibiotic agents on some of human pathogen bacteria.

Conclusion

In conclusion, *Holothuria tubulosa* is already known as a commercial sea cucumber species; however, more researchers are needed to explore the bioactive properties and functionality of this species in molecular basis to obtain improved sustainable production and utilization. The results of this study have shown that there can be a potantial source of natural antioxidant agents. Moreover, if the further researches are performed on this material, after isolation and purification processes of components, detailed antimicrobial activity tests should also be considered in investigations due to its bacteriostatic / bacteriocidal potential.

Acknowledgements

The present study was carried out with financial support of Scientific Research Fund of Canakkale Onsekiz Mart University (Project no FDK-2014-430).

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