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The antioxidant and antibacterial properties of fresh Mediterranean sea cucumber

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ABSTRACT

Mediterranean sea cucumber is one of the reach sources of undetected activities and active ingredients. The current study was conducted to find out the antioxidant and antibacterial properties of Mediterranean fresh sea cucumber, the selected pathogenic bacteria were as Staphylococcus aureus and Pseudomonas aeruginosa. The DPPH assay was used to detect the ability of fresh Sea cucumber extract to inhibit free radical cells activity, while microtiter plates assay was also used to evaluate the ability of fresh Sea cucumber extracts to reduce or inhibit the growth of selected Pathogenic Bacteria. Over all findings, S. aureus and P. aeruginosa were sensitive to all fresh Mediterranean Sea cucumber extracts, the highest Inhibition percentage of Sterile distilled water extract was as 100% against both of S. aureus and P. aeruginosa while the strongest inhibition percentage of Ethanol extract was as 82.38% against S. aureus and as 96.42% against P. aeruginosa, the maximum inhibition percentage of Methanol extract was as 97.50% against S. aureus and as 100% against P. aeruginosa, which means that there was a clear noticeable antibacterial activity for each extract regardless to the used solvent and utilized concentrations, and there wasn't any bacterial resistance. Thus, the results showed that the highest antioxidant efficacy was back to aqueous fresh Sea cucumber extract, which was as 59.22% at 5000 ppm and the IC_{50} value was 116.27, while the strongest antioxidant activity of Ethanol fresh Sea cucumber extract was as 48.94% at 2000 ppm and the IC_{50} value was at 63.37, ultimately the maximum antioxidant ability of Methanol fresh Sea cucumber extract was as 52.29% at 3000 ppm and the IC_{50} value was at 53.38. In conclusion, fresh Mediterranean Sea cucumber extracts were had antibacterial and antioxidant properties, and these properties varies according to the active ingredients nature, or species of pathogenic bacteria and its resistance activity.

1. Introduction

The aquatic commodities that have high economic value and potential properties for used as One pharmaceutical and nutraceutical products are Sea cucumbers, particularly the antibacterial and antioxidant properties [1]. Sea cucumbers are deluxe nutrients and foods, and have been used to heal kidney problems, rheumatism, impotence, reproductive disorders, joint pain, asthma, back pain,

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hypertension, constipation, wound injuries, cuts and burns [2]. By nutritional side, sea cucumbers contain low quantities of lipids (basically polyunsaturated fatty acids (PUFAs) and high quantities of protein (40-60%), in addition to the vitamins (e.g., A, B1, B2, and B3) and minerals (e.g., calcium, zinc, iron, and magnesium) [3]. Apart from these, Sea Cucumber Contains a group of bioactive ingredients, including phenolics (flavonoids and phenolic acids), triterpene Glycosides (Saponins), Proteins (Collagen and Peptides), Polysaccharides (Fucosylated Chondroitin Sulfate), Cerebrosides, and Sphingoids, which explain its potential activites, as Antioxidant , Antimicrobial, Anticancer , Anti Diabetic, anti inflammatory, antithrombotic, Anti Obesity, and Anti Hypertension Activities [4]. Sea cucumbers are also showed to have antibacterial activity [5], particularly against the pathogenic bacteria [6], thereby, Sea cucumbers in Previous studies as antibacterial agent reported that the ethyl acetate fraction in sea cucumbers can discourage the growth of Staphylococcus epidermidis and Propionibacterium acnes bacteria [7]. Thus, they could be as potential sources for modern antibiotics in view of multiple recent reports on the appearance of antibiotic resistant pathogenic bacteria resulting from the excessive and unregulated use of antibacterial agent [8].

In addition to that, several studies have found that many bioactive substances that have extracted from sea cucumber species are have beneficial properties, for instance antiangiogenic, wound healing, and antibacterial effects [9]. Scientifically, Sea cucumbers bioactive compounds have been proven to decrease free radicals and prevent various dangerous diseases that resulting from the excess free radicals [10]. Extracts of subtropical sea cucumbers can protect against reactive species reduced damage at the cellular level and they also known as antidotes against free radicals with positive effects on human health [11]. Over the past decade, Reactive oxygen species (ROS) and free radicals have attracted increasing attention. ROS, including hydroxyl radicals (OH*), superoxide anion radicals (-02*), singlet oxygen ('Or), and non-free radical species such as H2O, comprise various activated oxygen forms, exacerbation of these molecules lead to cellular injury and process of aging [12].

Moreover, biomolecules such as proteins, lipids, carbohydrates, and nucleic acids are damaged due to the ROS oxidative induction, this damage causes diseases including aging and cancer, more than 100 diseases have been implicated by the ROS, including heart disease, malaria, acquired immunodeficiency syndrome, stroke, arteriosclerosis, diabetes, and cancer [13]. Skin is one of the largest immune organs in the body of humans, and also presents the main epithelial barrier between the hostile environment and human body [14], increased risks of hospital acquired infections and lowering host immunity can be caused through painful skin traumatic injuries that are occurred by burns or wounds [15]. One of the opportunistic pathogens that are responsible for hospital acquired infections is Pseudomonas aeruginosa (*P. aeruginosa*) [16], Also, *Staphylococcus aureus* (*S. aureus*) is one of the most important pathogens that is related to antimicrobial resistance around the world [17], it can resist the other major antimicrobial classes, in addition to the almost of all lactams (e.g., flucloxacillin, oxacillin, and methicillin) [18]. Historically, the first resistant of *S. aureus* against the methicillin (MRSA) was reported in 1961 [19], therefore one of the most important global threats to general public health is antimicrobial resistance [20, 21].

The efficacy of antimicrobial drugs is reduced through the spreading of multidrug resistant (MDR) pathogens, thereby prolonging hospital stays thus increasing treatment costs and fatalities[17]. Molecular entities or molecular fragments, capable of independent existence are known as Free radicals, they include one or more unpaired electrons in an outer molecular orbital or atomic orbital [22], The increased formation of endogenous or exogenous origin of reactive oxygen and reactive nitrogen species is lead to oxidative stress due to antioxidant imbalance and pro oxidant that causes metabolism cellular damage [23]. The inflammation of cells, necrosis and apoptosis, damage to DNA base damage, DNA single stranded and double stranded breaks, DNA and

chromosomal aberration, lipid membrane, DNA and protein cross links, collagen structure and mitochondrial function can be increased. Many diseases such as cardiovascular diseases, chronic obstructive pulmonary disease, chronic kidney disease, neurodegenerative diseases and cancer are caused by the oxidative stress that means that oxidative stress plays an important role in these mentioned diseases [24], while the system that protects the organism against the harmful effects of free radicals, repairs or reduces the damage, is known as antioxidants, many diseases such as cancer, metabolic syndrome, atherosclerosis, malaria, Alzheimer's disease, rheumatoid arthritis, neurodegenerative diseases and preeclampsia. As It is known these diseases are caused by the mechanisms which increase the ROS that resulting from oxidative stress, so the ROS are positively related to the pathology of these diseases [25, 26]. The published work on Mediterranean Sea cucumber are really limited, however there is a study indicated into systematic review on sea cucumber farming and potential development, to prevent its extinction and limit its poaching in North-Eastern Atlantic and Mediterranean Sea, and did not involve any antioxidant or antibacterial activities of sea cucumber، The study was carried out and aimed to providing a comprehensive summary of the state of the art of farming practices of the European sea cucumbers that are considered as commercial fisheries target, this study was included a total of 34 original articles [27]. Moreover, there was no work detected from Libyan cost sources. Therefore, the current study aimed to screen the ability of fresh Mediterranean Sea cucumber to inhibit the growth of burn infection multi antibiotic resistant *Pseudomonas aeruginosa* and Staphylococcus aureus, free radical.

2. Methodology

2.1 Sea cucumber

Sample of fresh Mediterranean Sea cucumber was obtained from Misurata coast, through Tobactes club, Misurata Libya, and kept at -20 °C for the further study.

2.2 Preparation of Fresh Sea Cucumber Sample

The used part of the fresh sea cucumber was the whole body except the digestive system, the fresh sea cucumbers were cut into small pieces and extracted using maceration with three different solvents (Sterile distilled water, Ethanol, and Methanol), Concentration of extraction: 25g Sea cucumber/ 50ml Solvent. Extracts left for overnight in the shaker at room temperature, the crude extracts were filtered by filter papers (Round filter paper with 110mm pores diameter), and evaporated in an oven at 40 °C for seven days, aiming to remove remaining solvents, the delay that had happened in drying, was mostly due to small surface area of used tubes nozzles (Small diameters), and/or the use of the fresh sea cucumbers that were contained high amounts of physiological fluids, after evaporation amount of watery and alcoholic (ethanol and methanol) extracts were collected, the amount from the water solvent was more than that collected from alcoholic, after that each sample was diluted by adding 1ml of sterile distilled water and filtered using filter disk , All samples were kept at 4 C for further study [1].

2.3 Collection and preparation of Pathogenic Bacteria

Pathogenic Bacteria that used in this study were collected from the microbiology department, Misurata Medical Center, and they were isolated from burns cases. The selected strains were Staphylococcus aureus and Pseudonymous aeruginosa. Both pathogenic bacteria were sub-cultured in nutrient broth and agar to ensure their purity and activate them. Catalase test and gram stain were

carried out, the pure samples then were kept in fresh broth and agar media at 4 °C for the further experiments [28].

2.4 Evaluation of Antibacterial Activity of Sea Cucumber Extracts by Microtiter Plates Assay

Samples were tested against the selected pathogenic bacteria in Microtiter plate assay, following the method of Aween and others, Briefly, 100 µL of nutrient broth containing ±107 CFU/ mL was placed in the 96 well plate and 100 µL of diluted samples was poured into the wells, the plates were then incubated at 37 °C for 24 h. Optical density of bacterial growth was measured at 600 nm using Elisa plate reader (BIOTEK, BioTek Instruments, Winooski, VT, USA). Sample with nutrient broth without bacteria was used as negative control, and nutrient broth with pathogenic bacteria was used as positive control. This experiment was done in triplicate and growth inhibition percentage was calculated as mentioned in statistical analysis part [29].

2.5 Antioxidant Assay Using DPPH

Antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) were applied [30].

2.5.1 Sample Preparation

The prepared samples of fresh Sea cucumber extracts were exposed to a concentration process in five different concentrations (1000, 2000, 3000, 4000, and 5000 ppm) for each solvent, which mean there were five used concentrations for each used solvent, thereby, there were 15 samples, and 5 samples of used standard (Ascorbic acid) due to the use of the same five different concentrations too, last but not least, the extracts were exposed to DPPH assay to evaluate the fresh Sea cucumber antioxidant activity against free radicals [30].

2.5.2 DPPH Preparation

The 100 µg/ml of DPPH solution made by weighing 5 mg of DPPH and dissolve in 50 ml of methanol (96%) in a measuring flask [30].

2.5.3 Measurements of Samples

Measurement of blank antioxidant capacity was conducted by measuring 2 ml of DPPH mixed with 3 ml of methanol, incubated at 37 °C for 30 min. The maximum wavelength was then measured in the range of 510 to 520 nm by using Eliza reader (Biotek, Germany). The measurement of antioxidant capacity of the diluted fresh sea cucumber extracts were carried out by piping 1 ml of each extract separately. Afterwards, 2 ml of DPPH and 2 ml of methanol were added to concentrations, and then all samples were incubated at 37 °C for 30 min. Measurements were taken by Eliza reader at wavelength of 513 nm [30].

3.6 Statistical analysis

All experiments were done in triplicate, mean and standard division, percentage of inhibition and one way Anova were carried out using Minitab 18 series. Percentage of inhibition was calculated by the formula.

3. Results

3.1 Preparation of Fresh Sea Cucumber Sample

The fresh sea cucumber samples that had extracted were dried in an oven at 40°C until the solvents evaporated and kept at 4 °C for further study, as shown in Fig. 1.

Fig. 1. Evaporated fresh Mediterranean Sea cucumber samples

3.2 Collection and Preparation of Pathogenic Bacteria

The growth of the pathogenic bacteria were varies, depends on its species. During the preparation of the pathogenic bacteria, all bacteria samples showed turbidity in Nutrient broth media as a first sub-culture and then pure colonies on Nutrient agar as a second sup-culture, specifically colour, and shape as shown in Figure 2.

3.3 Antibacterial Activity Evaluation Using Microtiter Plates

Sea cucumber extracts with their different concentrations showed a range of antibacterial activity against the selected pathogenic bacteria *Pseudomonas aerogenes* and *Staphylococcus aureus*, these activities varied depending on used solvents, concentration, and incubation time.

The fresh Sea cucumber extracts that had extracted using sterile distilled water, which diluted by adding 1ml of sterile distilled water for each sample, achieved the highest activity levels as total inhibition (100%), after 12 to 24 hours of incubation against both tested pathogenic bacteria *S. aureus*, as shown in Table 1 and *Pseudomonas aeruginosa*, shown in Table 2.

The Sea cucumber extracts that had extracted using Ethanol, achieved lower activity levels as 78.36%, after 12 hours of incubation, and 82.38%, after 24 hours of incubation against S. aureus, as shown in Table 3., while achieved activity level of 96.42%, after 12 hours, and 93.37%, after 24 hours against *Pseudomonas aeruginosa*, as shown in Table 4.

Table 1
Percentage of antibacterial activity of fresh sea cucumber extracts that were extracted using sterile distilled water solvent against *S. aureus*.

Incubating time/h	Inhibition percentage
12h	100%
24h	100%

Table 2
Percentage of antibacterial activity of fresh sea cucumber extracts that were extracted using sterile distilled water solvent against *Pseudomonas aeruginosa*

Incubating time/h	Inhibition percentage
12h	100%
24h	100%

Table 3
Percentage of antibacterial activity of fresh sea cucumber extracts that were extracted using Ethanol solvent against *S. aureus*

Incubating time/h	Inhibition percentage
12h	78.36%
24h	82.38%

Table 4
Percentage of antibacterial activity of fresh sea cucumber extracts that were extracted using Ethanol solvent against *Pseudomonas aeruginosa*

Incubating time/h	Inhibition percentage
12h	96.42%
24h	93.37%

The Sea cucumber extracts that had extracted using methanol, also achieved lower activity levels than that achieved by using sterile distilled water, and they were as 96.68% percentage of inhibition, after 12 hours of incubation , and as 97.50%, after 24 hours of incubation , against *S. aureus*, as shown in Table 4.5, meanwhile achieved a high activity levels equal to sterile distilled water activity levels (100%), after 12 to 24 hours of incubation, against *Pseudomonas aeruginosa*, as shown in Table 6.

Table 5
Percentage of antibacterial activity of fresh sea cucumber extracts that were extracted using Methanol solvent against *S. aureus.*

Incubating time/h	Inhibition percentage
12h	96.68%
24h	97.5%

Table 6
Percentage of antibacterial activity of fresh sea cucumber extracts that were extracted using Methanol solvent against *Pseudomonas aeruginosa*.

Incubating time/h	Inhibition percentage
12h	100%
24h	100%

Compared to the study conducted by Ardiansyah and others, 16 Indonesian Sea cucumbers had inhibitory activity against three types of pathogenic bacteria that cause acne: *Propionibacterium acnes* (*P. acnes*), *Staphylococcus aureus* (*S. aureus*), and *Staphylococcus epidermidis* (*S. epidermidis*), by using Microtiter plate bioassay, different results were detected, and results showed that, there were Six species out of sixteen, had antibacterial activity against *S. aureus* [31]. In contrast to the current study, Rasyid and others conducted a study to evaluate the antibacterial activity of extracts from five sea cucumber species against a number of pathogenic bacteria including *S. aureus*, by using disc diffusion method, all of which displayed distinct activity profiles. Notably, the methanol extracts of *Holothuria leucospilota* (*H. leucospilota*) effectively inhibited the growth of all reference bacteria, yielding clear zone diameter, the ethyl acetate fraction of *Holothuria leucospilota* (*H. leucospilota*) was active against *S. aureus*, exhibiting a clear zone diameter of 7.2 ± 0.28 mm [32]. In addition to another study carried out by Sukmiwati and others to determine the antibacterial activity of *Holothuria atra* (*H. arta*) sea cucumber against *Pseudomonas aeruginosa*, by using disc diffusion method, the test results of inhibition of antibacterial activity based on inhibition zone diameter of sea cucumber *H. atra* methanol (50%) extract against *Pseudomonas aeruginosa* bacteria, and the obtained Inhibitory zone (mm) was 8.58±0.03 [33].

Through all the previous published works [31, 32, 33], no papers found on using Microtiter plates assay to examine the antibacterial activity of the sea cucumber extracts, but in generally, it can be said that the Sea cucumbers were had antibacterial activities, regardless to the used assay methods.

3.4 DPPH ASSAY

The DPPH solution that mixed with tested fresh Sea cucumber extracts showed a change in color after 30 mins of incubation at 37 °C, that change from dark violet to light violet for sea cucumber extracts and yellow for ascorbic acid, that would prove to the ability of fresh Sea cucumber extracts to reduce or inhibit the action of free radical cells, as shown in Fig. 3.

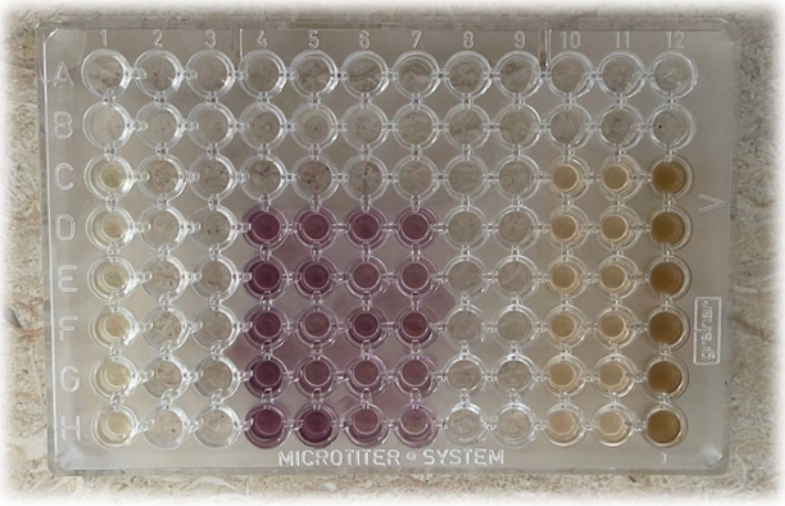

Fig. 3. Oxidation activity of fresh Sea cucumber extracts by DPPH test

All the different concentrations of fresh Sea cucumber extracts (1000, 2000, 3000, 4000 and 5000 ppm) showed varies levels of antioxidant activity, and this activity was compared to the activity of the used standard of Ascorbic acid as shown in Fig. 4.

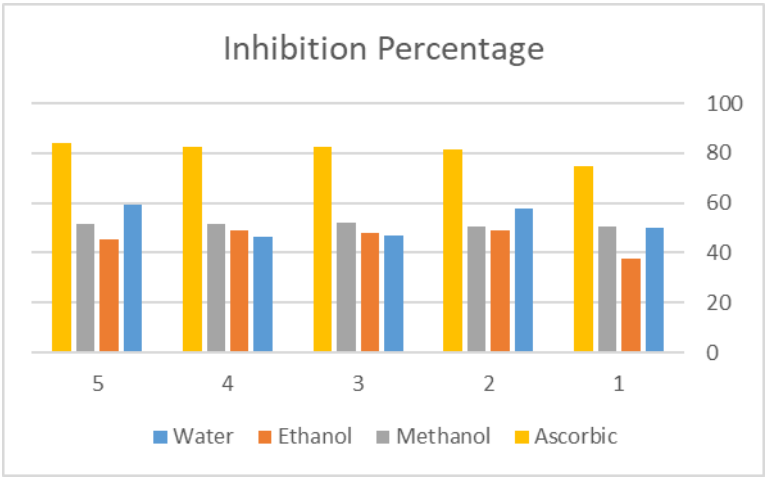

Fig. 4. Antioxidants activity of all fresh Sea cucumber extracts, compared to Ascorbic acid

The highest antioxidant activity of fresh Sea cucumber extracts that had extracted using sterile distilled water as a percentage of inhibition was obtained from 5000 ppm at 59.22%, followed by 2000 ppm at 57.83%, while 1000 ppm was at 50.10%, then 3000 ppm at 46.87%, and finally the lowest level was at 4000 ppm at 46.38%, as shown in Fig. 5, and it's IC_{50} value was at 116.27.

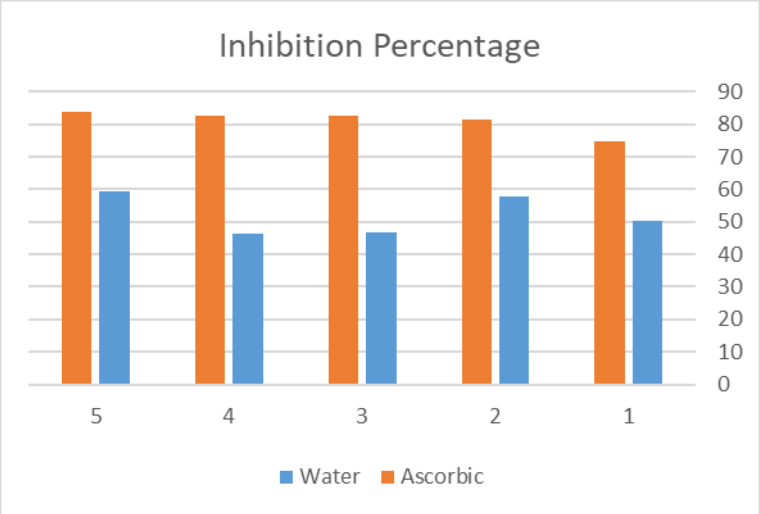

Fig. 5. Antioxidants activity of fresh Sea cucumber extracts that had extracted using sterile distilled water, compared to Ascorbic acid

In fresh Sea cucumber extracts that had extracted using ethanol as solvent, the highest antioxidant activity as a percentage of inhibition was obtained from concentration of 2000 ppm at 48.94%, followed by 4000 ppm at 48.72%, while 3000 ppm was at 48.12%, then 5000 ppm at 45.27%, and finally the lowest level was at 1000 ppm at 37.84%, as shown in Figure 4.6, and it's IC_{50} value was at 63.37.

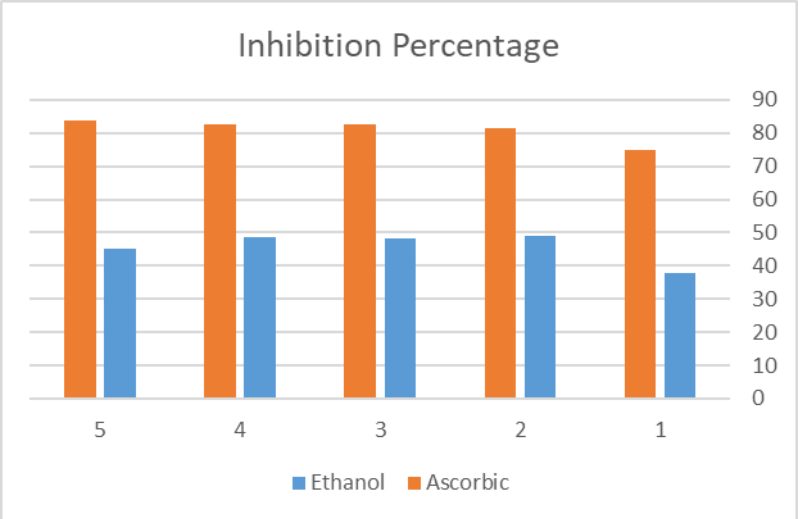

Fig. 6. Antioxidants activity of fresh Sea cucumber extracts that had extracted using ethanol as solvent, compared to Ascorbic acid.

While fresh Sea cucumber extracts that had extracted using methanol as solvent, showed that the highest antioxidant activity as a percentage of inhibition was obtained from a concentration of 3000 ppm at 52.29%, followed by 5000 ppm at 51.71%, while 4000 ppm was at 51.70%, then 2000 ppm at 50.63%, and finally the lowest level was at 1000 ppm at 50.52%, as shown in Figure 4.7, and it's IC_{50} value was at 53.38.

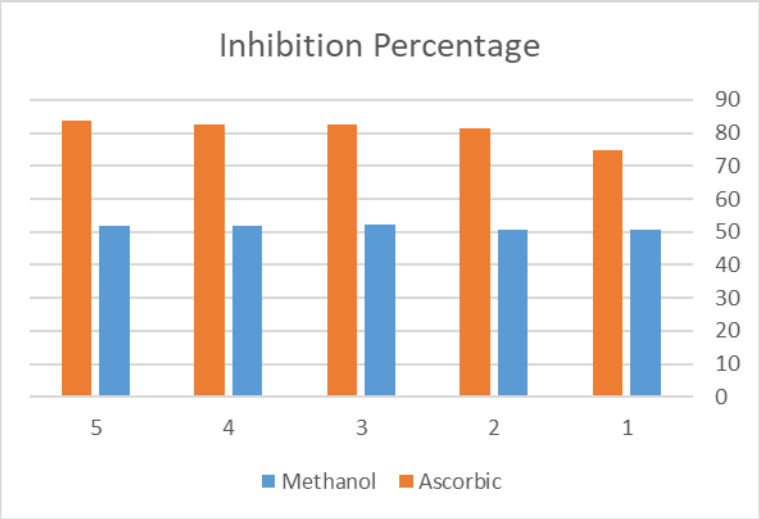

Fig. 7. Antioxidants activity of fresh Sea cucumber extract that had extracted using Methanol as solvent, compared to Ascorbic acid.

The Ascorbic acid antioxidant activity results were as followed, the highest antioxidant activity as a percentage of inhibition was obtained from a concentration of 5000 ppm at 83.90%, followed by 4000 ppm at 82.55%, while 3000 ppm was at 82.5%, then 2000 ppm at 81.35%, and finally the lowest level was at 1000 ppm at 74.84%, and it's IC_{50} value was at 88.84. After the came out of DPPH results, these results were used to calculate the values of IC_{50}, the highest value was obtained from aqueous extract and it was at 116.27, followed by the utilized the standard which was Ascorbic acid at 88.84 , while Ethanolic extract was at 63.37 , then ultimately the Methanolic extract was at 53.38 as sown in Table 7.

A study conducted by Nugroho and others, to measure antioxidant activity on samples of 21 species of Indonesian Sea cucumbers using DPPH method, and the revealed results showed that Holothuria atra (*H. atra*) sea cucumber has the strongest antioxidant activity and the percent of inhibition was 36.36 ± 1.58% [34], which was comparable to the results of this study, and the results showed that the fresh Mediterranean Sea cucumbers had higher levels of antioxidant activities (59.22%). In contrast a study conducted by Wulandri and others to evaluate the antioxidant activity of cultivated sea cucumber (*Holothuria scabra*) from Bali, Indonesia, the bioassay of antioxidant activity of sea cucumbers had done by using the ABTS (Decolorization Assay of Antioxidant Capacity Reaction Pathways) method, and the obtained results of antioxidant analysis of Holothuria scabra sea cucumber extract that had extracted by water at three different temperatures (60, 70 and 80°C) had an IC_{50} values of 991.92 ppm,1005.80 ppm, and 1593.27 ppm, respectively, while the researchers did not calculate the percentage of inhibition [1]. In addition to another study evaluated by Hossain and others to determine the antioxidant activity of sea cucumber, using DPPH method, particularly *Holothuria leucospilota* Sea cucumber would be mentioned and the used part body, was Sea cucumber without viscera (aqueous and organic extracts), gave DPPH value range of 3.91 to 5.44% [35], which was also comparable to the results of the current study, that showed the fresh Mediterranean Sea cucumbers had higher levels of antioxidant activities (59.22%).

The antioxidant effect of fresh Mediterranean Sea cucumber was considered more efficient when compared to other samples that tested in previous studies [34, 35], which indicated that fresh Mediterranean Sea cucumber showed higher antioxidant activity.

4. Conclusions

The fresh Mediterranean Sea cucumber samples proved to have antibacterial ingredients against burn infections bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and also proved to have properties that could effective against free radical cells.

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