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Applying a cultured *Brachionus plicatilis* crude extract as a novel source of natural medical bioactive compounds

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ABSTRACT

Brachionus plicatilis is the most common rotifer species that has been cultured for fish farming. However, all applicable studies of *B. plicatilis* have been concerned about its nutritional values in aquaculture; while there is little attempt to use it as a potential source for medical bioactive substances. Therefore, the study aimed to culture the rotifer *B. plicatilis* in sustainability with *Cyclotella* sp. (as feeds), to applying its extractions as antitumor, antimicrobial, and antioxidant. *B. plicatilis* crude had a significant impact on the cell growth inhibition of MCF-7 cells (breast cancer), where the maximum cell growth inhibition (91.53 %) was detected with 10 mg/ml of the extract. On the other hand, the Gram-positive organisms (*Staphylococcus aureus*, *Streptococcus mutans* and *Methicillin-Resistant Staphylococcus aureus*) were moderately sensitive for the *B. plicatilis* extract, and their inhibition zones (16, 13 and 11 mm, respectively) were smaller than that (24, 20, and 15 mm, respectively) produced by gentamycin (control). Otherwise, the activity of *B. plicatilis* extracts against Gram-negative organisms was zero except with *Salmonella typhimurium*, which produced a very small inhibition zone (8mm). Also, the rotifer *B. plicatilis* extract showed antioxidant activity, but their IC_{50} value was larger than IC_{50} that belongs to ascorbic acid, which means that the antioxidant ability of ascorbic acid is stronger than *B. plicatilis* crud extract sample. Therefore, *B. plicatilis* crud extract may have a promising future in the treatments of many diseases, including cancer, and bacterial infection.

INTRODUCTION

In light of the spread of diseases among the people, the testing of all-natural and other products as a treatment for these diseases has become an urgent necessity for researchers and stakeholders. As such experiments and studies may open new avenues and hopes for the introduction of new compounds in the pharmaceutical industry. Aquatic organisms are promising to produce natural compounds that can be used in this regard. Where, the aquatic organisms are subjected to a very wide variation of environmental conditions, such as ranging of temperature, pressure, nutrients, and the intensity of sunlight. So, these organisms need to generate diverse natural products to adapt and protect themselves from those condition **(Wali *et al*., 2019)**. Therefore, many unique natural products have been isolated from various aquatic organisms like tunicates (ascidians), sponges, soft corals, sea hares, nudibranchs, bryozoans, sea slugs, and rotifers **(Donia and Hamann, 2003; Haefner, 2003; Byun *et al*., 2009)**. The number of natural products extracted from the aquatic organisms increases rapidly, and now exceeds hundreds of newly discovered compounds every year **(Faulkner, 2002; Proksch and Müller, 2006).** Aquatic medical bioactive substances can be applied to treat life-threatening diseases/disorders such as cancer, AIDS, and cardiovascular diseases **(Pettit *et al*., 1991; Erdmann *et al*., 2008; Mayer *et al*., 2013).**

Cancer is one of the most fatal disease among humans, therefore many attempts and trials have been done to inhibition its progression (**Wali *et al*., 2019).** For this purpose, numerous compounds isolated from aquatic organisms have ability to control this disease by anti-proliferative activity or enhancers of apoptosis against cancerous cells **(Wali *et al*., 2019).** Also, one of the most critical threats is antimicrobial resistance, which decreases the effective role of antimicrobial for the treatment of many infectious diseases **(Indraningrat *et al*., 2016)**. In this scope, a lot of organisms, including aquatic invertebrates, that can synthesize or/and accumulate several inhibitory compounds to the microorganisms' growth **(Burkholder and Sharma, 1969).** On the other hand, oxidation is one of the essential processes during the metabolism in all living organisms, which results in many free radicals and other oxidizing reactive oxygen species (**Hancock *et al*., 2001)**. The presence of such free radicals causes many serious diseases such as cancers, stomach ulcers, Alzheimer's, arthritis **(Leanderson *et al*., 1997; Das *et al*., 1997; Vajragupta *et al*., 2000)**; therefore the natural antioxidants can act as free radical scavengers and prevent mentioned diseases **(Chang *et al*., 2007).**

While several studies have concerned to use rotifers as an important live food and protein source for many aquatic fish larvae in aquaculture (**Byun, *et al*., 2009)**, a few studies have been focused on the usage of rotifers as an origin of bioactive substances **(Rumengan, 2007; Lee, 2010; Rumengan *et al*., 2014).** However, rotifer species have an opportunity for medical bioactive. Where they can have absorbed several substances from their given feed or the surrounding environment, that able to relocate, amino acids, unsaturated fatty acids, minerals, vitamins, and antibiotics without pollutant effects **(Byun *et al*., 2009; Rumengan *et al*., 2014; Fembri *et al*., 2017).**

Brachionus plicatilis is a common brackish and marine water zooplankton, and it is the rotifer species that have been cultured in the large scale **(Arimoro, 2006)**. However, all applicable studies of *Brachionus plicatilis* have been concerned about its nutritional values in aquaculture, while there is no attempt to use it as a natural source of bioactive substances.

Therefore, the present study aimed to culture the rotifer *Brachionus plicatilis* in large scale using *Cyclotella* sp. as feed, to applying its extractions as a source of medical bioactive substances as antitumor, antimicrobial, and antioxidant, where the investigating of different bioactive substances may open new avenues for introducing novel aquatic compounds into the pharmaceutical industry.

MATERIALS AND METHODS

Isolation of *Brachionus plicatilis*

Samples were collected from Lake Manzalah at the connection point between the Lake with Mediterranean Sea (Boughaz El-Gamil Region) by filtrating large amount of water using 55µm plankton net. *Brachionus plicatilis* was isolated under tri-nuclear light research microscope (NEJY ML-2700) at magnifications of 40x and 100x using a digital micropipette to start its culture.

The mass culturing of *Brachionus plicatilis*

Cyclotella sp. was collected from Lake El-Manzalah and isolated under inverted microscope (ZEISS 1M4738) at magnifications of 100x and 400x, then the culture of *Cyclotella* sp. was initiated and grown in a ceramic pond with a water volume of 4 m^3 according to artificial medium. **Guillard (1975)** recommended from growing diatoms, brackish water medium (SWES). The culturing water was underground and adjusted by a commercial salt at a salinity of 17.4%, and water was sterile by heating at 80 ^oC before the experiment beginning. Water temperature was constant at 17.5 ^oC and day-light conditions with continuous aeration by 2 HP Air Compressor each interval time of 15 minutes. *Brachionus plicatilis* growth was started by 3 Org./ml in the *Cyclotella* sp. culturing pond when the growth of the cultured *Cyclotella* sp. had enhanced. *B. plicatilis* and *Cyclotella* sp. densities were counted every 5 days under light tri-nuclear research microscope (NEJY ML-2700) at magnifications of 40x and 100x.

The preparation of *Brachionus plicatilis* crude extracts

At the peak of the rotifer growth, 500 litres of the water pond was filtrated by plankton net of 100µm mesh size to collect *B. plicatilis*. The harvested rotifers were concentrated too much and washed by distilled water to remove the salts and any substance from the collective mass.

The wet rotifers were placed in Petri dishes which introduced to the oven at 50 ^oC for 24 hrs to evaporate the excess of water and converted wet concentrated rotifer organisms to the powder of dried rotifers attached on Petri dishes, which were scraped to collect the powder in Eppendorf's tubes for usage in later applications. For antitumor and antioxidant activities applications, approximately 7.36 g of the *B. plicatilis* powder was macerated with 14.5 ml of 70% aqueous ethanol. The powder was infused in ethanol for a week with genital shaking; solutions were filtered by Whatman 542 filter paper. The solvent was vaporized by using a rotary evaporator to get the soluble extracts **(Ballantine *et al*., 1987).** For antimicrobial activity application, aqueous extract was prepared. Approximately 2 g. of the *B. plicatilis* powder was macerated with 10 ml of distilled water. The powder was infused in water for a week with genital shaking; solutions were filtered by Whatman 542 filter paper.

Antitumor activity

Viability assay of crude *B. plicatilis* extract against MCF-7 (breast cancer) cells was tested by Holding Company for Biological Products and Vaccines (VACSERA), Cairo, Egypt, using functional assay of "3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)" protocol according to **Supino (1995)**.

Antimicrobial activity

The assessment of the antimicrobial activity of crude *Brachionus plicatilis* extract was tested by Regional Center of Mycology and Biotechnology (RCMB), Al-Azhar

University, Cairo, Egypt, using the diffusion agar technique. Well diameter; 6.0 mm and 100 µl of aqueous extract (200 mg/ml) of *Brachionus plicatilis* was tested using gentamycin (4 µg/ml) as positive control and distilled water as negative control according to **Valgas *et al*. (2007).**

Antioxidant assay

The screening of crude *Brachionus plicatilis* extract for antioxidant activity was tested by Regional Center of Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt, using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) for detect the scavenging of free radicals according to **Yen and Duh (1994).**

RESULTS AND DISCUSSION

The mass culturing of *Brachionus plicatilis*

At the beginning of the mass culture, *Cyclotella* sp. was inoculated in the culture pond, and when its density reached 1×10^3 cell/ml, *Brachionus plicatilis* growth was started by 3 Org./ml in the same pond of *Cyclotella* sp. Both *Cyclotella* sp. and *B. plicatilis* densities were continued to increase over time until *Cyclotella* sp. reached its highest density of 61×10^3 cell/ml, while *B. plicatilis* density was 21 Org./ml on the 10th day. After that, the density of *Cyclotella* sp. began to decrease until it reached 48.073 $\times10^3$ cell/ml on the 15th day, while the density of *B. plicatilis* continued to increase until reached its highest levels of 80 Org./ml on the 15thday (Fig.1). At this time, about 500 liters of the culturing pond was harvested using plankton net 100 um mesh-size, before the beginning of the decline phase of *B. plicatilis*, based on our preliminary experiments preceded this study. The collected mass of *B. plicatilis* was 171.2 and 9.36 g for wet and dry weight, respectively.

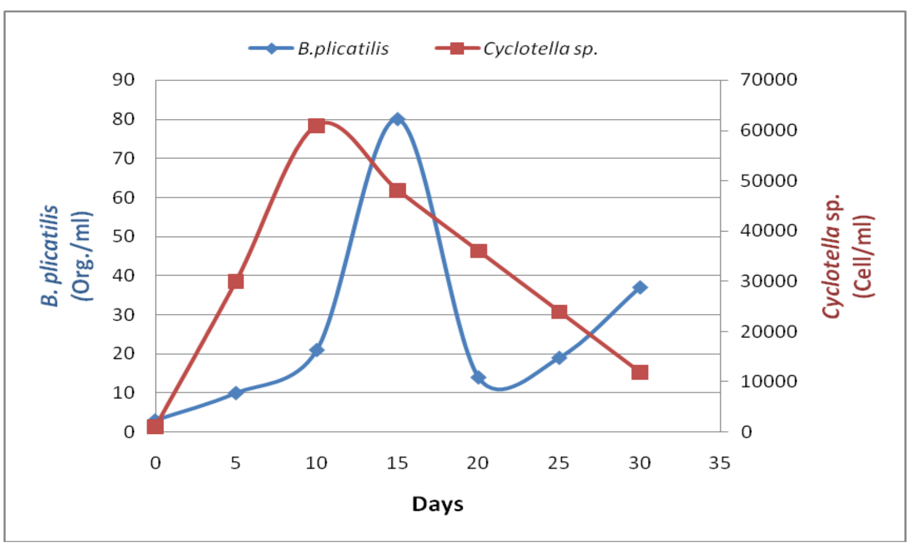

Figure 1 Growth densities of *Brachionus plicatilis* and *Cyclotella* sp. during the cycle of culturing.

In this study, *Cyclotella* sp. has been selected for the first time as food for getting a mass culture of *B. plicatilis*due because: (a) it was flourished at the same time and sites of *B. plicatilis*in Lake Manzalah, where the same salinity and appropriate temperature;

(b) cell diameter of *Cyclotalla*sp. acceptable for the mouth opening of *B. plicatilis*, where it ranges in size from 9 to 25µm (**Neumüller *et al*., 2002)**; (c) diatoms are easily digestible food for phytoplankton feeders due to the presence minute pores on their silica wall through which the digestive juices inflow inside the cell contents **(Fryer and Iles, 1972)**; its high nutritional value, where it is rich with protein, lipid, polyunsaturated and free fatty acids, phospholipid, sterol, and triglyceride classes **(Pahl *et al*., 2010).**

On the other hand, our highest density (80 Org./ml) of *B. plicatilis* is slightly low compared to that (93, 100 - 200, 100, 132 and 508 Org/ml) obtained by some previous studies (e.g. **Villegas, 1990; Park, 1991; Kongkeo, 1991; Alam and Shah, 2004; Freire *et al*., 2016)**, that fed *B. plicatilis* on *Tetraselmis tetrathele*, *Chlorella* sp., *Nannochloropsis oculata*, *Tetraselmis chui* and *Nannochloropsis limnetica*, respectively. However, these better results obtained by the previous studies may be attributed to their high initial number of *B. plicatilis* at the beginning of the culture, where they started their culture with 5 to 50 Org/ml, while our culture was started by only 3 Org/ml. Nevertheless, our highest density is slightly higher than that (43 to 47 Org/ml) obtained by some previous studies (e.g. **Villegas, 1990; Alam and Shah, 2004**), that fed *B. plicatilis* on *Chlorella* sp., *Nannochloropsis oculata* and *Isochrysis galbana*. These comparisons between our study and the other previous ones indicate that *Cyclotella* sp. is an acceptable food item for flourishing *B. plicatilis* in mass and sustainable culture.

Antitumor activity of crude *Brachionus plicatilis* extracts

In this study, the cytotoxic activity of *B. plicatilis* crude extract was tested against breast cancer cell line (MCF-7). The results showed significant variations in the cell growth inhibition of MCF-7 cells under the effect of the different concentrations of *B. plicatilis* crude extract (Fig. 2). The minimum cell growth inhibition (0.51 %) was observed under the effect of 156.25 µg/ml of the extracted sample. Then, the cell growth inhibition was gradually increased with increasing the extract concentrations. Where the maximum cell growth inhibition (91.53%.) was detected with 10 mg/ml of the extract. Generally, the crude extract of the rotifer *B.plicatilis* displayed cytotoxic activity on breast cancer cells with IC_{50} values of 967.85 µg/ml which able to kill the half number of tumor cells of MCF-7.

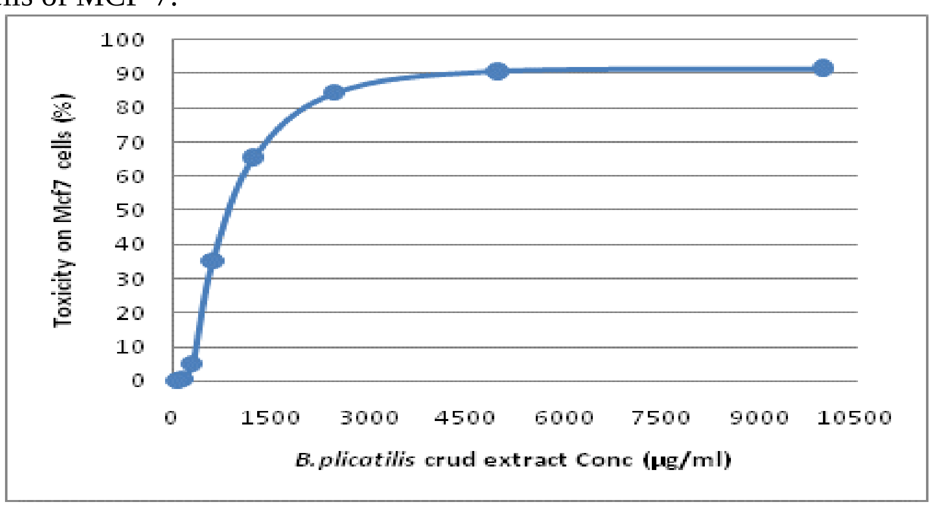

Figure 2. Effect of the crude *B. plicatilis* extract on MCF-7 cells at different concentrations.

These results are supported by many other previous studies, that recorded pharmacological bioactive compounds origin from aquatic invertebrates against cancer cells **(Wang *et al*., 1994; Huang *et al*., 2002; Leng *et al*., 2005; Song *et al*., 2008; Ning *et al.*, 2009; Pusphabai *et al*., 2010; Kim, 2011; Wang *et al.*, 2013; Ravikumar *et al*., 2010; Rajaram *et al.*, 2013; Umayaparvathi *et al*., 2014; García-Morales *et al*., 2016; Gomes *et al*., 2016; Correia-da-Silva *et al*., 2017; Ibrahim *et al*., 2017; El-Naggar *et al.*, 2020).** Furthermore, several novel antitumor compounds are isolated from many aquatic invertebrates and they have been under evaluation for human uses **(Wali *et al*., 2019)**.

Antimicrobial activity of crude *Brachionus plicatilis* extracts

The results of antimicrobial activity testes of *B. plicatilis* extract against several human and fish pathogens are including some Gram-positive pathogens and Gram-negative pathogens are shown in Table (1) and Fig. (3).

Table 1. Antimicrobial activity of crude *B. plicatilis* extracts against Gram-positive and Gram-negative pathogens.

Pathogenic Bacteria	Inhibition Zone (mm)	Control (Gentamycin)
Gram Positive Pathogens		
Staphylococcus aureus ATCC 25923	16	24
Streptococcus mutants RCMB 017 (1) ATCC 25175	13	20
Methicillin-Resistant *Staphylococcus aureus*	11	15
Gram Negative Pathogens		
Salmonella typhimurium RCMB 006 (1) ATCC 14028	8	17
Klebsilla pneumonia RCMB 003 (1) ATCC 13883	0	21
Proteus vulgaris RCMB 004 (1) ATCC 13315	0	25

The results show that all the tested Gram-positive organisms were moderately sensitive for the *B. plicatilis* extract and their inhibition zones were slightly smaller than that produced by gentamycin (control). Otherwise, the activity of *B. plicatilis* extracts against Gram-negative organisms were zero except with *Salmonella typhimurium*, which produced a smaller inhibition zone (8 mm) compared to that (17 mm) produced by gentamycin (control) (Fig. 3).

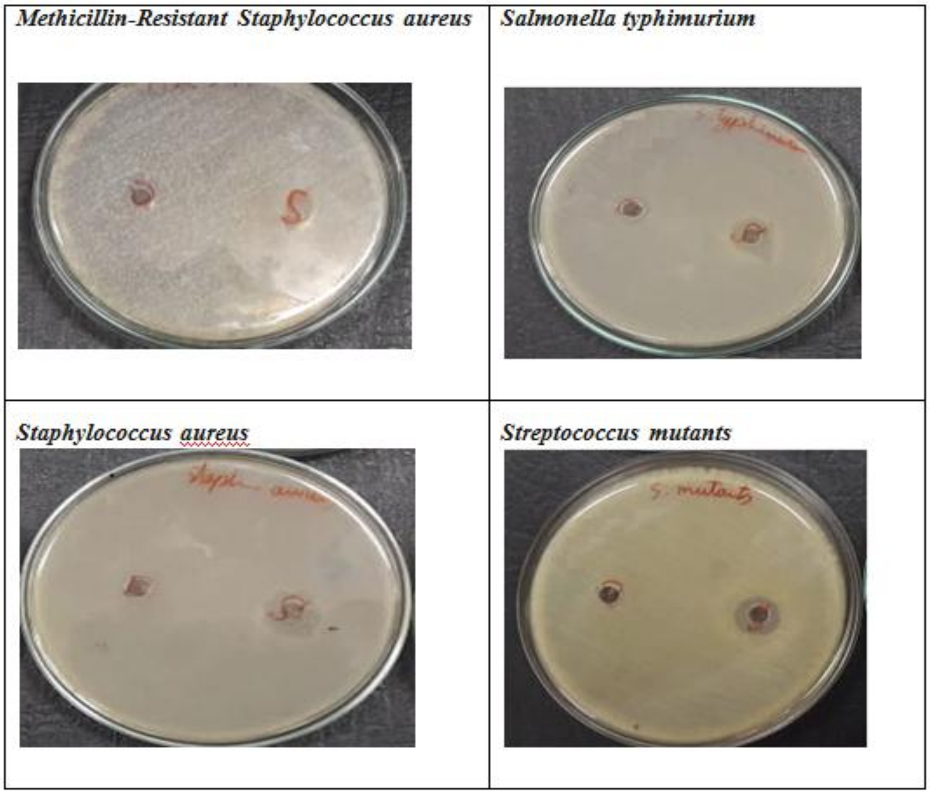

Figure 3 Hole zones of antimicrobial activities of crude *B. plicatilis* extract against three of Gram positive and one of Gram negative pathogens by Agar Well Diffusion Method.

These results of antimicrobial activity of *B. plicatilis* extract are supported by many studies that recorded the antimicrobial activity of different aquatic invertebrate's extracts **(Constantine *et al*., 1975; Ananthan *et al*. 2009; Natarajan *et al*., 2010; Kiran *et al.*, 2014; Ibrahim *et al*., 2017; El Samak *et al*., 2018 and Farisa *et al.*, 2019).** Furthermore, the antibacterial activity of the rotifer *B. plicatilis* crud extract against *Staphylococcus aureus*, *Streptococcus mutans* and *Methicillin-Resistant Staphylococcus aureus* (Gram-positive bacteria) was with slightly smaller inhibition zones (16, 13 and 11 mm, respectively) than that produce by gentamycin (24, 20 and 15 mm, respectively). However, these results are stronger antimicrobial activity than other studies that tested the antimicrobial activity of rotifer species extract against some Gram-positive bacteria. Where, **Rumengan *et al*. (2014)** reported that the rotifer *Brachionus rotundiformis* extract has not antibacterial activity against *staphylococcus aureus*, where its inhibition zone was equal to zero mm. Also, the same author recorded a very weak antibacterial activity of *B. rotundiformis* extract against *Bacillus subtilis* (Gram-positive bacteria) with a smaller inhibition zone (3.75 mm) than that produced by the control antibiotics, which was 24.16 mm.

On the other hand, the present results of antimicrobial activity of *B. plicatilis* crude extract against *Salmonella typhimurium* (Gram-negative bacteria) is stronger than that obtained by some other studies, that tested the antimicrobial activity of other rotifer species extracts against some Gram-negative bacteria. For examples; **Rumengan *et al*. (2014)** recorded the antibacterial activity of the rotifer *Brachionus rotundiformis* extract against *Escherichia coli* and *Vibrio cholera* (Gram-negative bacteria). However, their inhibition zones (4.66 and 3.5 mm, respectively) were very smaller than that produced by the control antibiotics, which was 23 mm. Also, **Farisa *et al*. (2019)** reported that the rotifer spp**.** extract has antibacterial activity against *Vibrio harveyi* (Gram-negative bacteria), but with a very smaller inhibition zone of 7.7 mm, compared to that (25.3 mm) produced by tetracycline (control).

Antioxidant of crude *Brachionus plicatilis* extracts

The study tested the antioxidant activity of the rotifer, *B. plicatilis* crude extract in corresponding to ascorbic acid standard to detect the scavenging ability of the rotifer extract by DPPH methanol solution. Fig. (4) showed that the antioxidant activity of ascorbic acid standard and IC_{50} belonged to a value of 14.2 µg/ml, while the antioxidant activity *B. plicatilis* extract was with a value of 255.6 µg/ml as IC_{50}. Therefore, the rotifer *B. plicatilis* extract showed an antioxidant activity (Fig.5), but their IC_{50} value was larger than IC_{50} which belonged to ascorbic acid. That means the antioxidant ability of ascorbic acid is stronger than *B. plicatilis* crud extract sample.

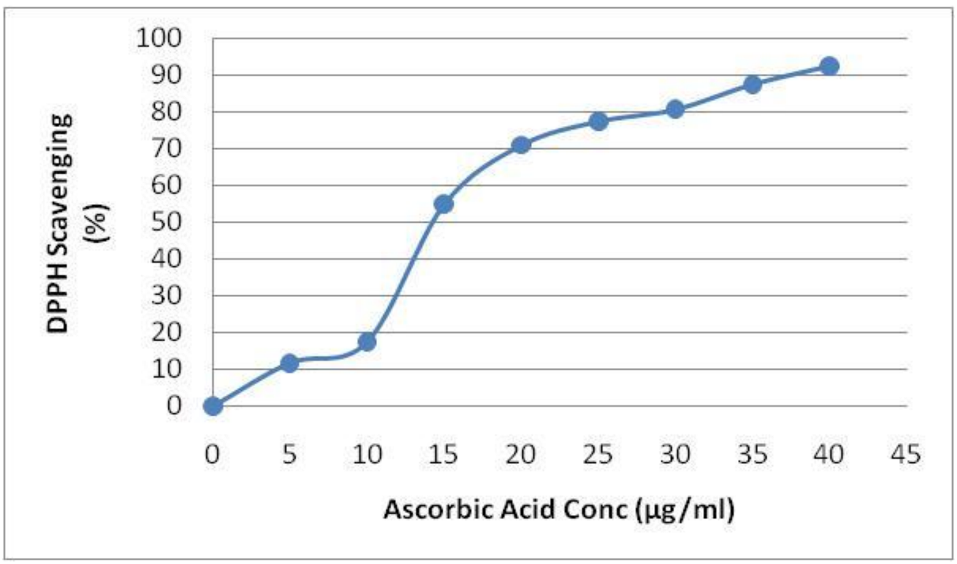

Figure 4. Evaluation of Antioxidant activity using DPPH Scavenging of Ascorbic acid reference standard.

The results of the antioxidant activity of the *B. plicatilis* crude extract are similar to other aquatic invertebrate's species, that appeared antioxidant activity by several studies (e.g. **Byun *et al*., 2009; Lee *et al*., 2010; Seradj *et al*., 2012; Umayaparvathi *et al.*, 2014; Ibrahim *et al*., 2017)**. Comparing to the antioxidant activity of other aquatic invertebrate's extracts, the antioxidant ability of *B. plicatilis* crude extract is stronger than the antioxidant activity of sponge, *Callyspongia crassa* extract which screened by **Ibrahim *et al*. (2017**). While, it is weaker than the antioxidant ability of oyster, *Saccostrea cucullata* crud extract, which screened by **Umayaparvathi *et al*. (2014).**

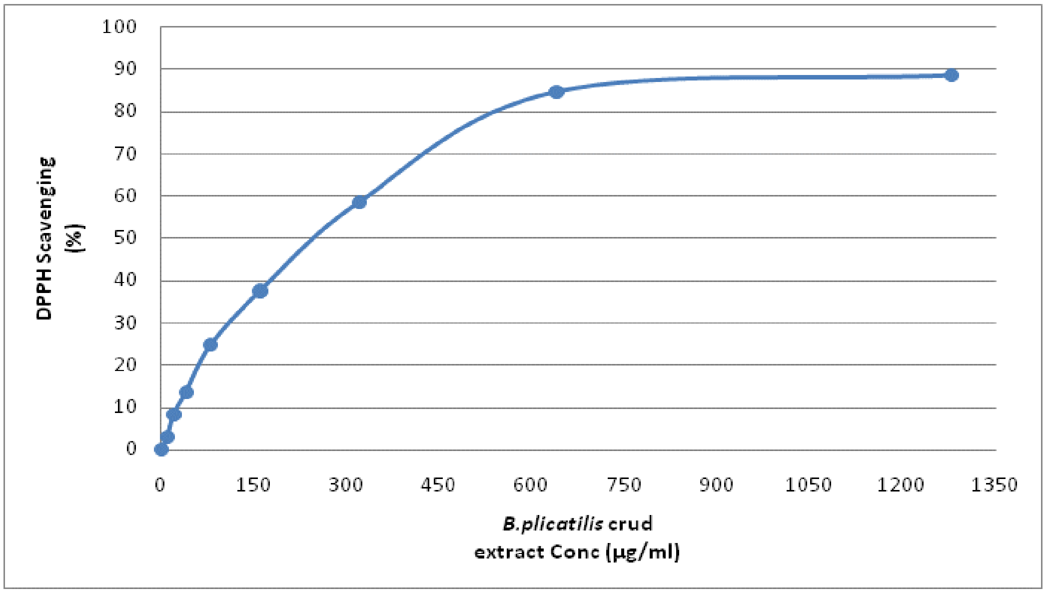

Figure 5. Evaluation of Antioxidant activity using DPPH Scavenging of *B. plicatilis* crud extract sample.

CONCLUSION

The current study aimed to culture the rotifer *Brachionus plicatilis* in large scale (using *Cyclotella* sp. as feed) to apply its extractions as a source of medical bioactive substances as antitumor, antimicrobial, and antioxidant. The study concluded that *Cyclotella* sp. is an acceptable food item for flourishing *B. plicatilis* in mass and sustainable culture. The crude extract of the rotifer *B. plicatilis* displayed cytotoxic activity on breast cancer cells with IC_{50} values of 967.85 µg/ml, which able to kill the half number of tumor cells of MCF-7. The antibacterial activity of the rotifer *B. plicatilis* crud extract was moderate against *Staphylococcus aureus*, *Streptococcus mutants* and *Methicillin-Resistant Staphylococcus aureus* (Gram-positive bacteria). While it had weak activity against *Salmonella typhimurium* (Gram-negative bacteria). The rotifer *B. plicatilis* extract also showed a moderate antioxidant activity compared to the antioxidant activity of ascorbic acid standard and IC_{50}. Therefore, *B. plicatilis* crud extract may be has the potential to provide future treatments for many important diseases, including cancer and bacterial infection.

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ARABIC SUMMARY

تطبيق مستخلص *Brachionus plicatilis* كمصدر جديد للمركبات الطبيعية الطبية النشطة بيولوجيا

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يعتبر "*Brachionus plicatilis*" من أكثر أنواع العجليات المستزرعة شيوعا، ومع ذلك فإن جميع الدراسات التطبيقية التى أجريت على هذا النوع اهتمت فقط بقيمتة الغذائية فى المزارع السمكية لذلك هدفت الدراسة الحالية علي اختبار تطبيق هذا النوع كمصدر للمواد الطبية النشطة بيولوجيا. لذلك قامت هذه الدراسة باستزراع هذا النوع بشكل مكثف ومستديم باستخدام نوع هام من الطحالب وهو "*Cyclotella* sp. " كغذاء هام له، ومن ثم تم عمل مستخلص من "*Brachionus plicatilis*" لقياس مدى نشاطه كمضاد للأورام وللبكتيريا وللأكسدة. وأوضحت الدراسة أن هذا النوع له فاعليه تثبيطية ضد خلايا أورام الثدى (MCF-7)، حيث تم الكشف عن أن تركيز ١٠ ملجم/مليلتر من المستخلص أدى إلى تثبيط نمو هذه الخلايا بنسبة ٩١.٥٣% وذلك كحد أقصى. من ناحية أخرى، كانت الكائنات الحية البكتيرية إيجابية الجرام (*Staphylococcus aureus* و *Streptococcus mutans*و *Methicillin-Resistant Staphylococcus aureus*) حساسة بشكل معتدل لمستخلص"*Brachionus plicatilis*"، وكانت نتائج مناطق تثبيطها لهذه الأنواع من البكتيريا هى (١٦ و ١٣ و ١١ مليمتر على التوالي) وذلك أصغر من قرينتها (٢٤ و ٢٠ و ١٥ مليمتر، على التوالي) التي نتجت عن استخدام الجنتامايسين، خلاف ذلك كان نشاط مستخلص"*Brachionus plicatilis*" ضد الكائنات الحية البكتيرية سالبة الجرام صفرًا باستثناء نوع "*Salmonella typhimurium*"، الذي أنتج منطقة تثبيط صغيرة جدًا (٨ مليمتر). أيضًا أظهر مستخلص"*Brachionus plicatilis*" نشاطًا مضادًا للأكسدة لكن قيمة "IC_{50}" له كانت أكبر من قرينتها الخاصة بحمض الأسكوربيك وهذا يعني أن قدرة مضادات الأكسدة لحمض الأسكوربيك أقوى من عينة مستخلص"*Brachionus plicatilis*". لذلك قد يكون لمستخلص"*Brachionus plicatilis*" الخام مستقبل واعد في علاج العديد من الأمراض، بما في ذلك الأورام السرطانية والأمراض البكتيرية.