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Controlling of Microbial Growth by Using *Cystoseira barbata* Extract

Neveen Abdel-Raouf, Hanaa M. Mohamed[*], Samah S. Mostafa[#], Ibraheem B.M. Ibraheem

Botany and Microbiology Department and [*]Genetic & Cell Biology Division ,Zoology Department, Faculty of Science, Beni-Suef University, Beni-Suef, 62514, Egypt.

MARINE algae contain a variety of bioactive secondary metabolites and several compounds have been derived from them for prospective development of novel drugs by the pharmaceutical industries. In this laboratory experiment *Cystoseira barbata* was isolated from Red Sea coastal water (Safaga, Egypt). It was evaluated due to its bioactivities potential. Where the algal extract proved a potent activity against bacterial and fungal strains ranged between medium and high suppression action. It showed that Gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* were more sensitive than Gram negative bacteria where *Serratia marcescens, Pseudomonas aeuroginosa* and fungus *Candida albicans.* Phyto-chemical analyses showed that *C. barbata* recorded the highest percentages of the flavonoids, phenols and saccharides compounds. Among the bioflavonoids determined Acacetin, Kaemp.3-(2-p-comaroyl) glucose, Rosmarinic and phenols were E-Vanillic, Benzoic and Ferulic were present in high percentages in the alga analyzed. The results indicated scope for utilizing this alga as a source of antibacterial and antifungal substances.

Keywords *: Cystoseira barbata,* Red Sea, Safaga, Antibacterial activity, Phyco-chemical analyses.

Introduction

Marine algae are widely distributed along the Egyptian Red Sea shore (Haroun et al., 1995; Mohamed et al., 2006 and Ibraheem et al., 2014). Among the marine flora, marine algae are rich sources of diverse bioactive compounds with various biological activities. Recently, marine algae detected as important source of novel bioactive substances. Many researchers have revealed that marine algal originated compounds exhibit various biological activities (Wijesekara, et al., 2010; Ibraheem et al., 2012 and Al-Saif, et al., 2014). During the last years, many studies have been made on biological activities of the marine algae and identified as potential sources of natural antioxidants (Matanjun et al., 2008 and Abdel-Raouf et al., 2015 a & b).

Marine organisms are sources of bioactive secondary metabolites with potent use in the development of new pharmaceutical agents (Abedin &Taha, 2008; Abdel-Raouf et al., 2008 and EL-Gamal, 2010). Kuniyoshi et al. (1985) reported that, diphenyl ether extracted from the green alga *Cladophora fascicularis* was found to inhibit the growth of *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. Other workers found that, algal extracts from Indian water *Dictyota dichotoma*, and *Padina gymnosora* were reported to be had activity against *Bacillus megatherium* and *S. aureus* (Rao et al., 1981). In this respect, Pesando & Caram (1984) reported that ethanolic extracts of *Zandariania prototypus, Cystoseira sricata* and *Cymbula compressa* inhibited the growth of different bacteria and fungi. On the national level, extracts of the Egyptian marine algae *Dictyota dichotoma, Dilophus fasciola* and *Cystoseira barbata* had antibacterial activities (El-Naggar, 1987 and Ibraheem et al., 2008). Antibacterial effects of hexane and methanol extracts of the marine algae *Mastocarpus stellatus, Laminaria digitata* and *Ceramium rubrum* on 12 marine and 7 prominent fish pathogenic bacteria were also reported **(**Dubber & Harder, 2007). Methanolic extracts of 32 macro-algae from the Atlantic and Mediterranean coasts of Morocco were estimated for the production of antibacterial compounds against *E. coli, S. aureus, Enterococcus faecalis* and *Klebsiella pneumonia* (Ibtissam et al., 2009**).**

The current work showed the potential of *C. barbata* isolated from Red Sea, as excellent sources of novel natural products with antimicrobial activities. Since the starting materials (algal extract) was devoid of any toxicity.

[#]Corresponding author email: samah.abdelgwad77@gmail.com, Tel.:+02 01068825644

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Materials and Methods

Studied area

The area investigated extends along Safaga coast on Red Sea, Egypt (Fig. 1). Safaga city located in the west coastal area of Red Sea shore between longitude 34°17`E and altitude 26° 06`N.

Fig. 1. Safaga location map, Rea Sea, Egypt.

Collection and identification of alga sample

Fresh and healthy marine alga *C. barbata* (Fig.2) was collected from the inter-tidal region between (0.2–2.5 m depths) along the Red Sea coast of Safaga, Egypt, during the period from April to June 2015. Collected sample was immediately brought to the laboratory in new plastic bags containing pond water to prevent evaporation. The algal material was washed thoroughly with tap water and distilled water to remove extraneous materials and shade-dried for 5 days and oven dried at 60ºC until constant weight was obtained, then was grind into a fine powder using an electric mixer and stored at 0ºC for future use. Algal species was identified (Aleem, 1993 and Coppejans et al., 2009).

Extraction for the selected algal species

Five hundred gm of *Cystoseira barbata* was mixed with 1000 ml of 99% ethanol and extracted in Soxhlet apparatus for 24 h and after evaporation in vacuum the extracts were stored as stock algal extract at -20°C until used **(**Krishnaveni et al., 2012).

Antimicrobial activity

Gram positive

For testing the anti-Gram positive activity, the following isolates including *Bacillus subtilis* (RCMB 01001 69-3), *Staphylococcus aureus* (RCMB 010027) and *Micrococcus luteus* (RCMB 01001 76-9) were selected.

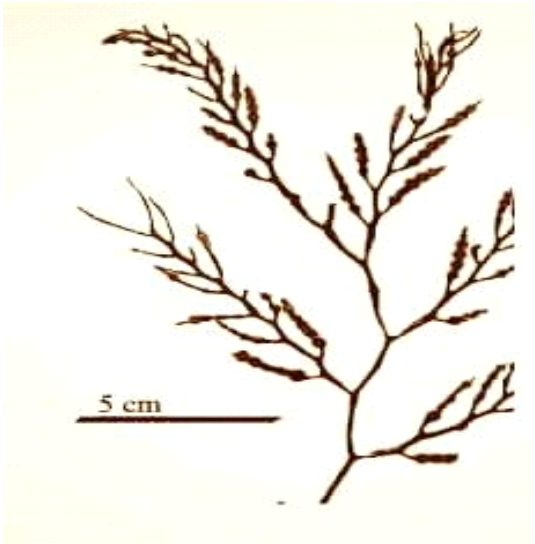

Fig. 2. Photographs of Cystoseira barbata.

Gram negative

For testing the anti-Gram negative activity, the following isolates including *Escherichia coli (RCMB 01002 52-6), Pseudomonas aeruginosa (RCMB 01002 43-5), Serratia marcescens (RCMB 01002 75b-8), Salmonella typhi (RCMB 01002 15-4), Vibrio* sp. and *Aeromonas hydrophila* were selected.

Fungi

For antifungal activity, the unicellular *Candida albicans* (RCMB 05036), was used for this purpose.

Antimicrobial activity by disc diffusion method

In the present study, antibacterial activity was determined against the above bacteria using the paper disc assay method (El-Masry et al., 2000). Whatman No. 3 filter paper disc of 6-mm diameter was sterilized by autoclaving for 15 min at 121°C. The sterile discs were saturated by 20µl with different concentrations (100, 200, 400 and 500 mg/ml) to become loaded by 2,4,8, and 10 mg for each disc, respectively. Agar plates were surface inoculated uniformly from the broth culture of the tested microorganisms. In all cases, the concentration was approximately 1.2 X 10^8 CFU/ml. The impregnated discs were placed on the Muller Hinton medium suitably spaced apart and the plates were incubated at 37°C for 24 h. Ethanol was used as a negative control while commercial antibiotic discs (Norfloxcin 10 mg/disc) were used as a positive control. The above procedure is allowed for fungal assays but expects the potato dextrose agar media instead of nutrient agar media. The plates were incubated at 25^0C for 48 h in the case of investigated fungus species. The diameter of the growth inhibition halos caused by the ethanolic extract of marine organism was measured by a ruler and expressed in millimeter. All the assays were carried out in triplicate.

Chemical analysis of Cystoseira barbata

HPLC analyses of phenolic compounds, polysaccharides and flavanoids

Phenolic compounds and polysaccharides in the ethanolic extract of marine specimens were determined according to method described by Goupy et al. (1999) and Zielinski et al. (2014), respectively. The detection were conducted using high performance liquid chromatography (HPLC Agilent 1200 series) equipped with Quaternary pump, Auto sampler, column compartment set at 35°C, multi wavelength detector set at 330 nm, 280 nm.

Statistical analysis

All results are presented as the mean ± SD. Statistical analysis was carried out by using one-way ANOVA (Duncan, 1957). Statistical significance was considered at $p < 0.05$.

Result

Ethanolic extract of the brown alga *C. barbata* was tested against some pathogenic Gram positive and Gram negative bacteria. The results of primary screening test are summarized in Table 1. *C. barbata* extract exhibited different antibacterial activities and inhibited all tested bacteria and fungus except *Micrococcus luteus*. For Gram negative bacteria, the maximum antagonistic activities of the *C. barbata* ethanolic extract (12.16 mm) were observed against *Serratia marcescens*, followed by *Pseudomonas euroginosa* (9.83 mm, respectively) at application of 10 mg/disc of the alga extract. On the other hand, for Gram positive bacteria, the maximum inhibition zone of the ethanolic extract of *C. barbata* was recorded against *B. subtilis* (16.00 mm**)** at application of 10 mg/disc of the alga extract. Additionally crude extract of *C. barbata* recorded a medium antagonistic activity against fungus *Candida albicans* **(**10.33 mm**)** at application of 10 mg/disc of the alga extract (Fig.3).

HPLC of the *C. barbata* ethanolic extract (Table 2) revealed the presence of 20 flavanoids in the alga extract. Also there are three flavanoids; Acacetin, Rosmarinic and Kaemp.3-(2-p-comaroyl) glucose were recorded high level (1677.166, 462.657, 128.137 ppm, respectively). The HPLC analysis revealed also a presence of 20 phenolic compounds in the alga ethanolic extract (Table 3). Further among the different phenols maximal levels were observed with E-Vanillic (139 ppm) as compared to other phenols followed by Benzoic (128 ppm). On the other hand, the HPLC analysis revealed that, 10 saccharides were present in the alga extract, among these saccharides: glucose (3.61%) and mannitol (3.46%) (Table 4).

Discussion

The paper disc susceptibility test of ethanolic extract from *Cystoseira barbata* was shown in Table 1. *Cystoseira barbata* extract demonstrated a good antimicrobial activity against gram-positive strain (*Bacillus subtilis* and *Staphylococcus aureus)* and gram-negative strains (*Serratia marcescens* and *Pseudomonas aeuroginosa)* in addition to the unicellular fungus *Candida albicans*. It was showed that the algal extract was more effective against Gram (+) than Gram (–) bacteria. The algal species belonging to genus *Cystoseira* possess a wide variety of compounds with different biological activities (Kolsi et al., 2015). This result disagree with Salvador et al. (2007) and Alghazeer et al. (2013**)** who said that *C. barbata* has no activity against *Pseudomonas* sp. The present study revealed that, ethanol is better solvent for extract the active substances of *Cystoseira barbata*, it was agreed with Souhaili & Faid (2016) who reported that *Cystoseira tamariscifolia* would possess an active antibacterial, antifungal and antimycotoxins extractible compounds in the ethanol which must be isolated and identified for possible uses in the biotechnological and or therapeutic domain.

TABLE 1. Antimicrobial activities of the *C. barbata* ethanolic extract (inhibition of growth expressed as mean ± SD).

Pathogen	Diameter of inhibition zone, mean ± SD					
	Cystoseira barbata concentrations mg/disc (Tested sample)				Norfloxcin (positive) control	Ethanol (negative) control
	2 mg/ disc	4 mg/ disc	8 mg/ disc	10 mg/ disc	10 mg/disc	10 µl/disc
Gram positive bacteria						
Staphylococcus aureus	6.66±0.57	6.66±0.67	10.33±0.57	13.30±1.15	30.60±01.15	00
Micrococcus luteus	**ND**	**ND**	**ND**	**ND**	33.30±02.88	00
Bacillus subtilis	11.33±0.57	11.33±2.30	14.00±1.00	16.00±1.00	26.00±04.04	00
Gram negative bacteria						00
Serratia marcescens	ND	ND	ND	12.16±0.57	26.00±01.00	00
Salmonella sp.	07.00±1.00	07.50±0.50	08.66±1.15	09.66±2.50	27.00±05.00	00
Vibrio sp.	06.83±0.76	07.00±1.00	07.33±0.57	07.83±0.28	25.00±12.66	00
Aeromonas hydrophila	07.50±0.50	08.00±1.00	08.00±1.00	08.00±1.00	28.00±15.50	00
Pseudomonas aeuroginosa	06.66±0.57	06.83±0.80	7.66±3.78	9.83±1.15	33.00±02.8	00
Escherichia coli	ND	ND	07.33±1.15	08.66±1.15	11.00±01.00	00
Fungi						
Candida albicans	7.60±0.57	9.00±1.00	10.33±0.57	**10.33±0.57**	20.00±01.15	00

Data are expressed as the mean ± standard deviation (SD) of three replicates. Represent the statistical comparisons between alkaloid extract and positive control by using ANOVA ($p<0.05$). ND: not detectable

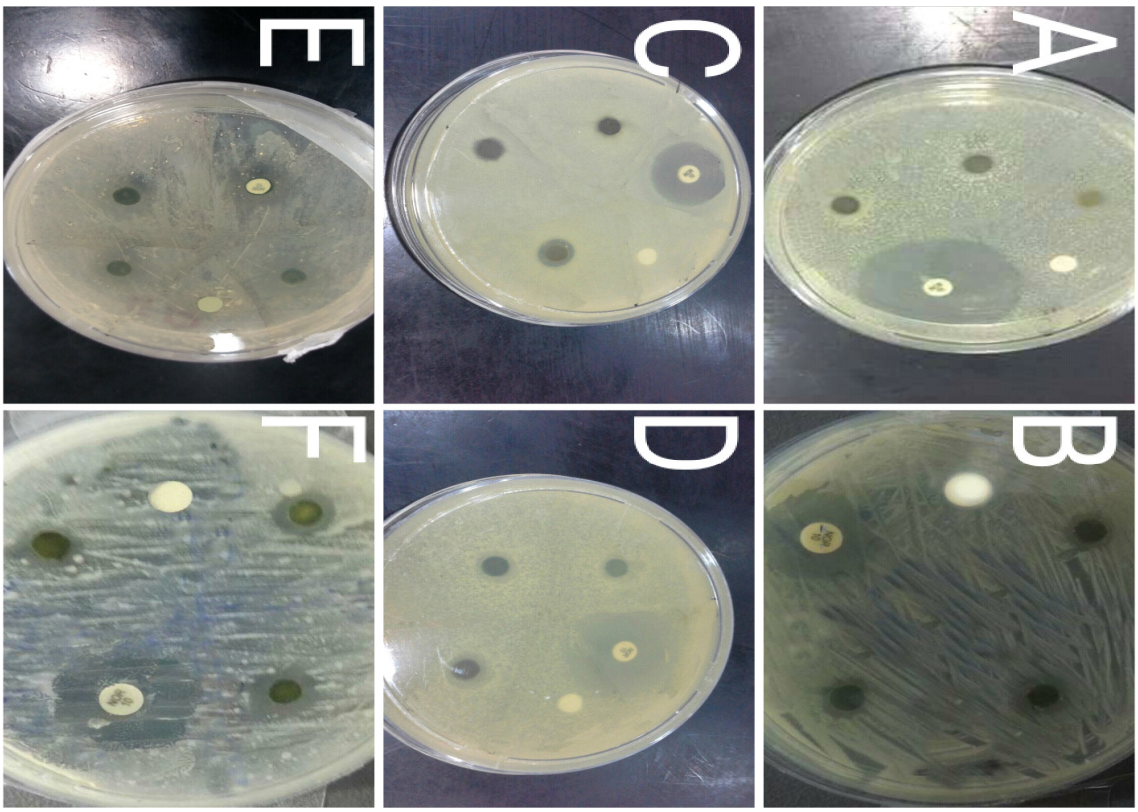

Fig. 3. Inhibition zones obtained by the ethanolic extract of *Cystoseira barbata* against *Micrococcus luteus* (A), *Candida albicans* (B), , *Pseudomonas aeuroginosa* (C), *Serratia marcescens* (D), *Staphylococcus aureus* (E) and *Bacillus subtilis* (F).

TABLE 2. HPLC analysis for flavonoid contents of *Cystoseira barbata* isolated from Safaga Sea shore during April to June 2015.

Flavonoids contents	ppm
Luteo.6-arabinose8-glucose	24.904
Apig.6-rhamnose8-glucose	24.666
Apig.6-glucose8-rhamnose	10.071
Naringin	29.213
Luteo.7-glucose	9.042
Hespirdin	39.317
Rutin	9.675
Quercetin-3-o-glucoside	15.931
Rosmarinic	**128.137**
Apig.7-o-neohespiroside	7.256
Kamp3,7-dirhamoside	14.747
Quercetrin	93.287
Quercitin	6.504
Kaemp.3-(2-p-comaroyl)glucose	**462.657**
Naringenin	6.049
Hespirtin	55.784
Kampferol	35.672
Rhamnetin	20.875
Apigenin	27.085
Acacetin	**1677.166**

TABLE 3. HPLC analysis for phenolic contents of *Cystoseira barbata* isolated from Safaga Sea-shore during April to June 2015.

Phenolic contents	ppm
Pyrogallol	53.5
Gallic	7.83
4-Amino-benzoic	48.2
Protocatchuic	8.4
Catechein	59.8
Catechol	3.98
Chlorogenic	18.4
Epicatechein	8.16
P-OH-benzoic	57.7
Caffeine	17.2
Caffeic	9.1
Ferulic	**71.6**
Iso-Ferulic	11
E-Vanillic	**139**
Benzoic	**128**
Ellagic	46.9
Coumarin	50.1
3,4,5-methoxy-cinnamic	6.11
Salycilic	76.8
Cinnamic	8.02

TABLE 4. HPLC analysis for saccharides contents of Cystoseira barbata isolated from Safaga Sea-shore during April to June 2015.

Saccharides contents	%
Glucuronic	0.78
Stachyose	1.33
Sucrose	0.66
Glucose	**3.61**
Xylose	2.76
Rhaminose	0.51
Mannose	0.52
Fructose	1.62
Mannitol	**3.46**
Sorbitol	0.23

Cox et al. (2010) reported that, organic solvents always have higher efficiency in extracting anti-bacterial compounds compared to water as extracting solvent. They said that the antimicrobial activity of red and green seaweed extracts significantly increased when ethanol and acetone were used as extraction solvents. Earlier studies have suggested that antimicrobial activity depends on the type of extraction solvent used, but also on algal species **(**Sunilson et al., 2009 and Boonchum et al., 2011). It is in contrast with those reported by Mhadhebi et al. (2012) who found that, antibacterial activity of methanolic extracts of some *Cystoseira* species revealed high antagonistic activity against tested bacteria more than petroleum ether extracts of *C. sedoides* and *C. crinita* which exhibited a moderate antibacterial activity against *Escherichia coli*. These results are in agreement with those reported earlier for isolating antibacterial substances as hydroquinones, sesterpenoids, phenols, brominated phenols and polyphenols from species of Chlorophyceae, Phaeophyceae and Rhodophyceae (Faulkner, 2002).

HPLC analysis of flavanoids, phenols and saccharieds for the studied alga Cystoseira barbata were carried out on extract to separate the compounds that were responsible for the inhibition tested bacteria. The inhibition activity for the ethanolic extract of Cystoseira barbata may be attributed to the presence of three flavonoid compounds, namely: acacetin, rosmarinic and Kaemp.3-(2-p-comaroyl) glucose or to the presence of the phenolic E-Vanillic or to the presence of the free saccharides glucose and mannitol which recorded high level in the alga

extract compared to the other flavonoid, phenolic and free saccharides which present in relatively low amounts. These compounds responsible for the antimicrobial activity and assume that the active compounds could be at least partly, lipophilic halogenated compounds. Phenols have been identified in several algal species as biologically active compounds (Vairappan, et al., 2001). Many compounds of marine algae show anti-bacterial activities as polysaccharide (Laurienzo, 2010), lyengaroside (Ali et al., 2002), polyhydroxy lated fucophlorethol (Sandsdalen et al., 2003), bromophenols (Oh et al., 2008), guaiane sesquiterpene (Chakraborty & Lipton, 2010), lactone malyngolide (Cardelina et al., 1979) cycloeudesmol (Sims et al., 1975), polyphenolic compound (Devi, 2008), halogenated compound (Vairappan, 2003) and quinone metabolite (Horie et al., 2008). Flavonoids comprise a large group of naturally compounds widely distributed in the algae and some of these compounds have been reported to contain various and potent biological activities including anti oxidative tissue protective and tumoristatic effects as well as the inhibition of hepatic cholesterol biosynthesis (Krant et al., 2005; Kim et al., 2007; Matanjun et al., 2008 and Volk, 2009). Indeed, marine algae contain polyphenols, carotenoids and flavonoids as antioxidants, protect the body's tissues against oxidative stress and associated pathologies such as cancer and inflammation (Tapiero et al., 2002).

According to the previous reports, marine algae are rich sources of fiber, minerals, proteins, antioxidant and bioactive compounds. A number of bioactive compounds which have been isolate from marine algae include sulphate polysaccharides (laminarin and fucoidans), polyphenol (such as phlorotannins), carotenoid pigments (such as fucoxantin and astaxanthin), sterols and mycosporine-like amino acids (MAAs) (Airanthi et al., 2011 and Gupta & Abu-Ghannam, 2011). Bromophenol has been reported as biofuntion of antimicrobial compounds was to found in the red marine algae (Oh et al., 2008). In addition to those mentioned above, the mechanism of phenolic compounds influences the cell wall and cell membranes of microorganism. Moreover, it can interfere with the membrane function such as destroy the electron transport, nutrient uptake, protein, nucleic acid synthesis and enzyme activity. These active phenolic compounds might be several invasive targets which could lead to inhibition of bacteria (Gupta & Abu-Ghannam, 2011). In this study, the active compound presented in crude extracts might be interacting synergistically for bacterial inhibition.

Conclusion

In conclusion, the result of the present study confirm that marine alga *Cystoseira barbata* have potential source of bioactive compounds against various human pathogens, which can be used as natural non-toxic preservative and may be more acceptable to consumers. Further work is needed to identify the active compounds and role of antibacterial activity of this marine alga.

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التحكم في النشاط الميكروبي بواسطة أستخدام مستخلص طحلب سيستوسيرا باربانا

نيفين عبدالرؤوف محمد، هناء محمود محمد*، سماح سعيد مصطفي وإبراهيم برعى محمد إبراهيم
قسم النبات والميكروبيولوجي و*شعبة الوراثة - قسم الحيوان- كلية العلوم - جامعة بني سويف - بني سويف - مصر.

الطحالب البحرية تحتوي على مجموعة متنوعة من النواتج الأيضية الثانوية النشطة بيولوجيا ، حيث أُستخدمت العديد من هذه المركبات لتطوير الأدوية الجديدة من قبل الصناعات الصيدلانية. في هذه التجربة المختبرية تم جلب الطحلب البني **سيستوسيرا باربانا** من المياه الساحلية في البحر الأحمر (سفاجا، مصر)، حيث تم أستخلاص المركبات الحيوية المحتمل وجودها بأستخدام المذيب العضوي (إيثانول ٩٩٪). أثبتت النتائج أن مستخلص الطحلب له نشاطاً قوياً ضد السلالات البكتيرية والفطرية الممرضة والتي تراوحت بين المثبط المتوسط والعالي. وأظهرت النتائج أن البكتيريا الموجبة لصبغ جرام **باسلس ساطلس واستافيلو كوكس** أكثر حساسية من البكتيريا السالبة لصبغ جرام. وأظهرت التحاليل الكيميائية أن طحلب **سيستوسيرا باربانا** يحتوي على نسب عالية معنوياً من المركبات الفلافونويد والفينولات والسكريد ، من بينها: بيوفلافونويدز (أكاسيتين، Kaemp.3-2 ف-كوماروبل) الجلوكوز، روزمارينيك والفينولات كانت E-فانيليك، البنزويك و فيروليك. وأظهرت النتائج مجالا لاستخدام هذا الطحلب كمصدر للمواد المضادة للبكتيريا والفطريات.