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Inhibitory Potential of *Eucheuma denticulatum* (N.L.Burman) F.S. Collins & Hervey Against Selected Foodborne Pathogens

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Seaweed is reported to have antimicrobial properties that can possibly prevent the food from microbial invasion and spoilage. *Eucheuma denticulatum* (N.L.Burman) F.S. Collins & Hervey is a very abundant seaweed in Leyte province, wherein it is a common part of the human diet. The common problem in food handling, especially street food, is the fast rate of spoilage due to improper handling and packaging. One way of preventing the incidence of food infection and intoxication due to improper packaging of food is the use of packaging material that can inhibit microbial growth. The study was undertaken to determine the antimicrobial potential of *E. denticulatum* against common food pathogens. Six pathogenic bacteria – namely, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli* O157:H7 – plus a yeast (*Candida albicans*) and a mold (*Aspergillus flavus*) were used as test organisms of the study. Agar well diffusion assay was used to test the inhibitory potential of *E. denticulatum* extract against the selected pathogens. The ethanolic extract exhibited inhibitory potential against the test microorganisms except for Gram-positive bacteria *B. cereus* and *S. aureus*. The extract is more effective against Gram-negative bacteria *S. typhi* and *E. coli*. *C. albicans* exhibited the highest mean zone of inhibition of 16.11 mm at 75% extract concentration, and *A. flavus* has the least zone of inhibition of 2.78 mm. The observed inhibitory action of the ethanolic extract is a promising indication that *E. denticulatum* is a potential source of bioactive compounds that can be used as a natural food preservative.

Keywords: agar well diffusion assay, antimicrobial, seaweed, *E. denticulatum*, foodborne pathogens

INTRODUCTION

In recent years, there has been an increase of microorganisms resistant to antibiotics that are typically used in the treatment of some diseases (Fendrick and McKellar 2014). The statistic is alarming now as numerous antibiotics and drugs resistant to human pathogenic cases were reported globally (Nascimento *et al.* 2000). Despite the breakthrough of pharmacological industries in producing new antibiotics, nonetheless, there is still an elevation of resistance towards these antibiotics by microorganisms (Nascimento *et al.* 2000).

Marine organisms are potential sources of bioactive primary and secondary metabolites potential for the development of new pharmaceutical agents, and many of these substances have been demonstrated to

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possess interesting biological activities (Pandithurai *et al.* 2015). Seaweeds were reported to produce a wide variety of bioactive secondary metabolites as antimicrobial, antifeedant, antihelmintic, and cytotoxic agents; its bioactive substances included alkaloids, polyketides, cyclic peptide, polysaccharide, phlorotannins, diterpenoids, sterols, quinones, lipids, and glycerols (Cabrita *et al.* 2010). It is considered as the actual producers of some bioactive compounds with high activity (Shimizu 1996). Thus, marine organisms appear as an efficient alternative source of new drugs, and algae have been extensively documented to provide a rich source of primary and secondary metabolites (Mendes *et al.* 2013).

Considering that seaweed contains bioactive compounds and part of the human diet, this study was conducted to evaluate the potential antimicrobial property of *Eucheuma denticulatum* (N.L.Burman) F.S. Collins & Hervey, a common edible seaweed in the Philippines. *E. denticulatum* was once called *E. spinosum*; it has shorter axes covered from base to apex with spinous determinate (ramuli) or indeterminate branchlets arranged irregularly and in whorls confined to the apical portion of the branches (Ganzon-Fortes *et al.* 2011). *E. denticulatum* is a red alga with the potential to trace because it contains primary and secondary metabolites such as hydrocarbons; it has been used commercially for cosmetics, medicines, organic fertilizers, and textiles (Sugrani *et al.* 2019). This seaweed is very abundant in Leyte province as it is being cultivated in the area for food and carrageenan powder processing. In this context, the study focused on the determination of the inhibitory potential of ethanolic extract of *E. denticulatum* against selected foodborne pathogens, which are Gram-positive bacteria *Bacillus cereus* and *Staphylococcus aureus*; Gram-negative bacteria *Salmonella typhi* and *Escherichia coli* O157:H7; yeast *Candida albicans*; and mold *Aspergillus flavus*.

MATERIALS AND METHOD

Procurement of Samples

Fresh *E. denticulatum* was bought at the wet public market of Baybay, Leyte, Philippines, which was sourced out from Duwahon Island, Bato, Leyte (Figure 1). It was packed in plastic bags and transported immediately to the laboratory of the Department of Food Science and Technology (DFST), College of Agriculture and Food Science (CAFS), Visayas State University (VSU), Visca, Baybay City, Leyte. About 300 g of the said seaweed was submitted to the Department of Fisheries, VSU-Tolosa Campus, Tolosa, Leyte for seaweed sample identification.

Figure 1. Fresh *Eucheuma denticulatum* (N.L.Burman) F.S. Collins & Hervey from Dawahon Island, Bato, Leyte, Philippines.

Preparation of Extracts

The *E. denticulatum* sample was washed with sterile filtered seawater to remove any epiphytes and other contaminants and then rinsed with sterile distilled water. About 1000 g of *E. denticulatum* was dried in the cabinet dryer for 2–3 h at 60 °C until more than 80% moisture was removed. The dried *E. denticulatum* was weighed and was added with 60% ethanol at 1:2 ratio. The mixture was homogenized using an electric blender and the homogenate was allowed to stand for 48 h to allow the solvent to further act on the sample. The homogenate was then filtered using a previously sterilized muslin cloth and the filtrates were collected into a Florence flask. The filtrates were concentrated using a rotary evaporator (ROTAVAPOR) with a 40–60 °C temperature setting at the College of Veterinary Medicine (CVM), VSU, Visca, Baybay City, Leyte. The produced extract was equally divided into four parts and used as the base treatments: 100% seaweed extract (T1), 75% seaweed extract with 25% solvent (T2), 50% seaweed extract and 50% solvent (T3), and 25% seaweed extract with 75% solvent (T4).

Preparation of Test Organism

The antimicrobial potential of *E. denticulatum* extract was tested against selected foodborne pathogens and the microbial analysis was done at the CVM, VSU, Visca, Baybay City, Leyte. The test organisms used include two Gram-positive bacteria (*B. cereus* ATCC 14579 and

S. aureus ATCC 25923), two Gram-negative bacteria (*E. coli* O157:H7 and *Salmonella typhi* ATCC 14028), yeast (*C. albicans* ATCC 10231), and mold (*A. flavus* ATCC 15517). The stock cultures were maintained in a 1% glycerol broth and kept inside the refrigerator (4 °C) prior to use. Twenty-four-hour (24-h) old stock culture was mixed with physiological saline and the turbidity was corrected by adding sterile physiological saline (9.5 mL 1% H_2SO_4) until a McFarland turbidity standard of 0.5 ($1.5 * 10^{-8}$ CFU mL^{-1}) was attained.

Determination of Antibacterial and Antifungal Activity

In vitro antibacterial activity was determined using Mueller-Hinton agar (MHA), while *in vitro* antifungal activity was determined using Sabourauds dextrose agar (SDA).

The antimicrobial activity was carried out using the agar well diffusion method. Overnight stock culture was transferred to sterile Petri plate with solidified 15 mL MHA for bacteria, 15 mL SDA for yeast, and 15 mL SDA with 14% tartaric acid for mold. Afterward, it was spread with a sterile swab to create a lawn. About seven equidistant wells with 6-mm diameter were made in each plate with a sterile cork borer. Among the seven wells, four wells were filled with 0.2 mL of different treatment of seaweed extract using sterile pipettes (Table 1). The center well was the positive control (ciprofloxacin for bacterial plates and theoconazole for fungal plates), and the remaining two wells were filled with 60% ethanol and distilled water. The plates with pathogenic bacteria were incubated at 37 °C for 18–24 h and 24–72 h for plates with pathogenic fungi. After the incubation period, the plates were closely examined for the inhibitory effect of the ethanolic extract against the pathogens. The inhibitory potential of the extract was assessed based on the development of the zone of inhibition around the well. The diameter of the zone of inhibition was measured in millimeters using a ruler. Each experimental result was determined by the average of three replicates.

Table 1. Concentrations of seaweed extract used as treatments of the study.

Treatments	Concentration
T_1	100%
T_2	75%
T_3	50%
T_4	25%
T_5 (control)	Ciprofloxacin/theoconazole
T_6 (control)	60% ethanol
T_7 (control)	Distilled water

Experimental Design

The experiment used a completely randomized design composed of four treatments at different levels of seaweed extracts (100, 75, 50, and 25% w/v) and three controls (ciprofloxacin/theoconazole, 60% ethanol, and distilled water) per test microorganisms (Table 1). The experiment was replicated thrice.

Statistical Analysis

The software STATISTICA 8.0 was used for statistical analysis of the data. Univariate analysis of variance was used in testing the significance of the differences in the diameter of the zone of inhibition per microorganism using *E. denticulatum* extracts. This was followed by Tukey's test to determine the significant difference among treatments. All procedures were performed at a 95% confidence level.

RESULTS AND DISCUSSION

The inhibitory action of *E. denticulatum* ethanolic extract is summarized in Table 2. Statistical analysis shows that the different test organisms have a different response to the inhibitory action of *E. denticulatum* ethanolic

Test organism	Mean zone of inhibition (mm)				
	Concentration per well				
	100%	75%	50%	25%	Tioconazole / Ciprofloxacin
C. albicans	14.6667^{de}	16.1111^{d}	13.0000^{de}	12.0000^{de}	22.3333^{c}
A. flavus	9.8889^{e}	2.7778^{f}	3.2222^{f}	2.7778^{f}	33.6667^{a}
B. cereus	0.0000^{f}	0.0000^{f}	0.0000^{f}	0.0000^{f}	27.2222^{bc}
S. aureus	0.0000^{f}	0.0000^{f}	0.0000^{f}	0.0000^{f}	27.5556^{b}
S. typhi	13.2222^{de}	10.6667^{d}	10.6667^{d}	10.7778^{d}	23.3333^{bc}
E. coli O157:H7	13.6667^{ed}	11.3333^{ed}	11.7778^{ed}	10.5556^{e}	22.8889^{bc}

Values with the same letter are not significant with each other; level of significance: $p \leq 0.05$

extract (Table 2). The extract showed inhibition against pathogens. The development of the zone of inhibition around the colony of the selected pathogens is a positive indication of the inhibitory potential of seaweeds and their capability to synthesize bioactive secondary metabolites inhibitory to the pathogens.

Results showed that ethanolic extract is inhibitory against selected pathogens except for Gram-positive bacteria *B. cereus* and *S. aureus*. The ethanolic extract is more effective against Gram-negative bacteria (*S. typhi* and *E. coli*) (Figure 2). This result agrees with the findings of El-Sheekh *et al.* (2014), wherein four different algal chloroform-methanol extracts were more effective against gram-negative bacteria than to Gram-positive bacteria. It is well-known that a thick peptidoglycan layer characterizes Gram-positive bacteria in its outer cell wall and this might have resisted the entry of the active inhibitory molecules, hence attributed to the less susceptibility. This also implies the choice of solvent used for extraction as per Pushparaj *et al.* (2014) – the more substantial extraction capacity of methanol could have produced a more significant number of active constituents or secondary bioactive compounds responsible for antibacterial activity. Moreover, Redelman *et al.* (2012) account that low alcohol concentration could not promote antimicrobial stresses known to affect *S. aureus* biofilm formation. Nevertheless, the noted antibacterial activity implies the probable presence of bioactive compounds like fucoidan, phlorotannins, chrysophaentin, and lactones (Shannon and Abu-Ghannam 2016; Wei *et al.* 2015; Castillo *et al.* 2015; Plaza *et al.* 2010; Kuznetsova *et al.* 2003). The result of the study also in agreement with the findings of Sugrani *et al.* (2019) to the inhibitory potential against *E.coli*; however, inconsonance inhibitory zone results of protein fraction from *E. denticulatum* at 9.56 mm against *S. aureus* bacteria for 24-h incubation. Before 24 h and after 48 h, the incubation period – the diameter inhibitory zones of protein fraction – still did not reach its optimum and decreased, respectively, as protein compounds of *E. denticulatum* is considered bacteriostatic, which can only inhibit bacteria but does not kill them.

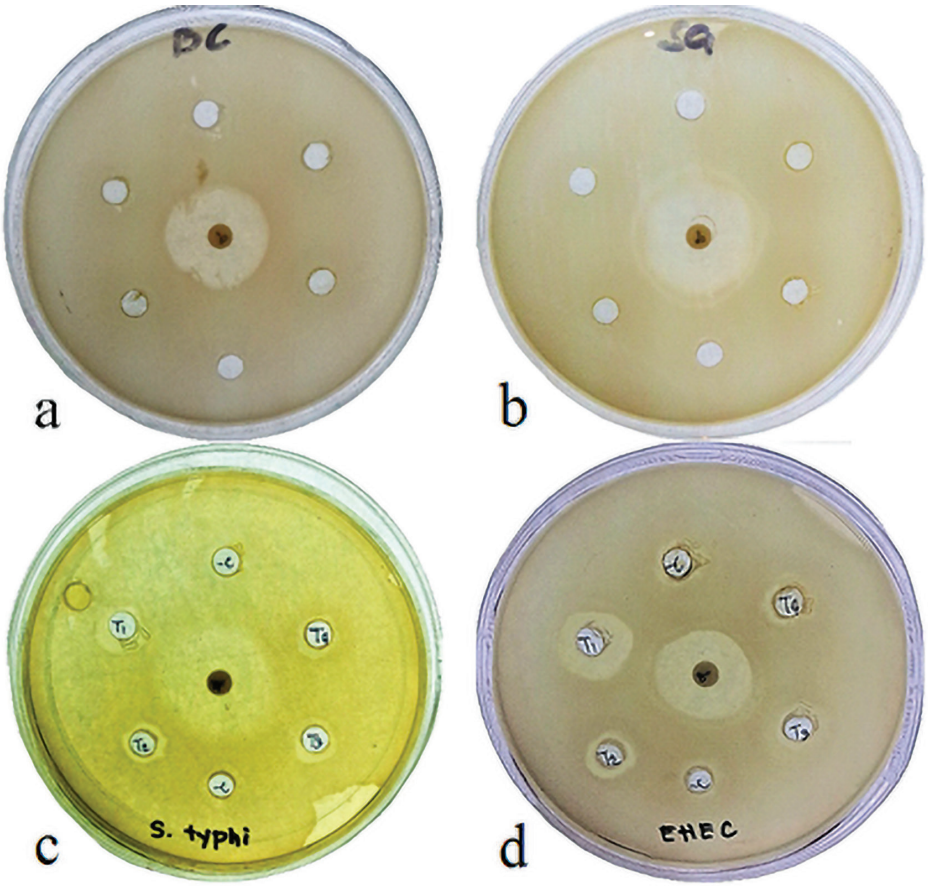

Figure 2. Antibacterial activity of *E. denticulatum* extract against a) *B. cereus*, b) *S. aureus*, c) *S. typhi*, and d) *E. coli* O157:H7.

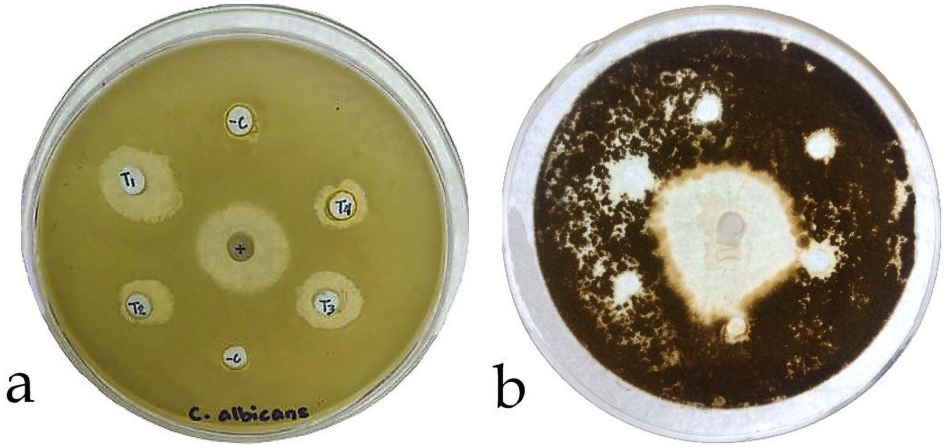

Figure 3. Antifungal activity of *E. denticulatum* extract against a) *C. albicans* and b) *A. flavus*.

The antifungal activity of *E. denticulatum* against *C. albicans* and *A. flavus* is presented in Figure 3. The *C. albicans* exhibited the highest mean zone of 16.11 mm inhibition at 75% extract concentration (Table 2). This result is in accordance with the findings of El-Sheekh *et al.* (2014) using *Sargassum ramifolium* extract, with only antifungal activity observed in *C. albicans* with a 15-mm zone of inhibition. The *E. denticulatum* extract also exhibited antifungal activity against *A. flavus* with a mean zone of inhibition ranging from 2.77–9.88 mm (Table 2). Zone of inhibition against *A. flavus* is far from the findings of Aruna *et al.* (2010) as the methanolic extract showed the maximum activity (56 mm) from 200 mg of *Ulva lactuca* against *Aspergillus niger* and minimum (4 mm) by 50 mg of *Ulva reticulata* against *Aspergillus flavus*. Methanolic extract of *K. alvarezii* exhibited 30 ± 3.7mm zone of inhibition followed by *Sargassum wightii* of 32 ± 2.8 mm against *A. flavus* at 100 mg concentration (Aruna *et al.* 2010). Shannon and Abu-Ghannam (2016) reported that methanol was determined to be most effective for brown seaweed, but using methanol only on green and red seaweed extracts had significantly lower to no antimicrobial activity than brown seaweeds. However, the potency of the green and red seaweeds increased significantly when combined ethanol and acetate were used as the solvent. Furthermore, the antifungal activity of *E. denticulatum* against *C. albicans* and *A. flavus* implies the probable presence of terpenic substances and other bioactive compounds are responsible for its antifungal activity.

The capability of *E. denticulatum* to inhibit the growth of selected pathogens is a positive indication that *E. denticulatum* is a potential natural food preservative. The presence of bioactive compounds is concluded with respect to the observed zone of inhibition. The European Union also issues directives on food-approved solvent systems and residual limits administered by the European Food Safety Authority; in such, Commission Directive 2010/67/EU sets out the maximum acceptable residues for acetone, hexane, and ethanolic extracts. The acceptable concentrations (retention factor) are given as not more than 500 mg kg^{-1} acetone, 25 mg kg^{-1} hexane, and 500 mg kg^{-1} ethanol. This correlates to the treatments used and it could be signifying almost no retained solvent in the extract.

CONCLUSION

The *E. denticulatum* ethanolic extract has inhibitory potential against *S. typhi*, *E. coli* O157:H7, *C. albicans*, and *A. flavus*. However, there was no prevalent inhibitory potential of *E. denticulatum* against Gram-positive bacteria *B. cereus* and *S. aureus*. Different concentrations of extracts may or may not have significantly affected the zone of inhibition (mm) exhibited by the extracts against the selected foodborne pathogens. Nevertheless, *E. denticulatum* has the ability to inhibit Gram-negative bacterial growth and has the potential to be used as natural preservatives. Moreover, *E. denticulatum* has the potential to be used as active antimicrobial packaging directed toward the reduction of surface contamination of processed and prepared foods, with the aim to prevent food infection and intoxication outbreaks.

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