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Antimicrobial, Antioxidant and Cytotoxic properties of *Eupatorium stoechadosmum* Hance extract for effective use in Oral care products

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Eupatorium stoechadosmum Hance is an indigenous medicinal plant with a pungent aroma and belongs to the family Asteraceae. The present work aimed to evaluate the antimicrobial and time-kill, antioxidant and cytotoxic properties of *Eupatorium stoechadosmum* Hance extract for effective use in oral care products. Crude extract (CE) was obtained from the leaves of *E. stoechadosmum* macerated with pure ethanol. A fractionated extract of *E. stoechadosmum* was established using hexane (F-Hexane), ethyl acetate (F-EtOAc) and ethanol (F-EtOH), respectively. Antimicrobial activity test against oral pathogens, *Streptococcus mutans* and *Candida albicans* were performed by disk diffusion, broth microdilution method and time-kill assay. Antioxidant activity was carried out using DPPH and ABTS assay. Cytotoxicity of the extract was analyzed through MTT assay using normal human gingival fibroblasts. The F-EtOH showed the strongest activity against *S. mutans* and *C. albicans*, minimum inhibitory concentration (MIC) and minimum concentration required to kill these pathogens were the same at 1.56 mg/mL and 3.13 mg/mL, respectively. The time-kill of F-EtOH against *S. mutans* and *C. albicans* was dose-dependent and completed with 4 x MIC within 2 and 3 h, respectively. F-EtOH also exhibited strong antioxidant activity with DPPH and ABTS scavenging activities. No cytotoxic effects on human gingival fibroblasts were recorded with F-EtOH in amounts up to 1000 µg/mL. This study demonstrated that F-EtOH of *E. stoechadosmum* exhibited strong antimicrobial and antioxidant activity, as well as low cytotoxicity. Therefore, it can be used as an ingredient for natural oral care products, to treat infections of *S. mutans* and *C. albicans*

.**Keywords: antimicrobial, antioxidant, cytotoxicity, *Eupatorium stoechadosmum* Hance, oral pathogens**

INTRODUCTION

Oral infections are a serious health problem throughout the world and are known to have significant health and economic impacts on populations around the world. The World Health Organization (WHO) has recognized the importance of control and prevention of oral diseases. The mouth is a major source of infections that can lead to oral disease and damage one's overall health (Okuda and Ebihara, 1998). *Streptococcus mutans* is a major component of oral bacteria and the main pathogen of dental caries (Hujoel et al. 2018). Caries represent a bacterial infection with the highest incidence of all oral diseases (Caufield, 2005). Caries infections can cause significant amounts of pain and lead to inflammation of the tissue that surround the tooth, *tooth loss*, infection and the formation of abscesses, all of which can result in bad breath (Laudenbach and Simon, 2014). *Candida albicans* is the most predominant fungal pathogen found in the human mouth, and it is known to be the main cause of oral candidiasis (Sardi et al. 2013). *C. albicans* infection can form a biofilm with *Streptococcus* bacteria, especially *S. mutans*. The infections can develop into dental plaque, which can then lead to caries, gingivitis, periodontitis and halitosis (Wang et al. 2012).

Therefore, prevention and treatment of oral infections that are known to cause dental caries, periodontitis, gingivitis and other related oral diseases can be achieved by reducing or eliminating oral pathogens. Today, the most commonly applied form of oral hygiene is homecare that involves the act of tooth brushing and the use of mouthwash. These actions have been shown to significantly reduce the amounts of oral pathogens (Van Den et al. 2008). Most oral care products contain chemicals, especially chlorhexidine, which have been found to effectively control plaque while preventing both gingivitis and periodontitis (Masadeh et al. 2013). However, evidence has shown that long-time use of these chemicals can cause certain side effects such as oral irritations, the staining of teeth, increases in the formation of calculus, alterations in taste perception, dry mouth and

unusual or unpleasant taste (Ggari and Kavani, 1995; Shrada, 2017). Triclosan, used as an ingredient in mouthwash, has been reported to combine with free chlorine in water resulting in a toxic substance (Roule et al. 2005). For this reason, it has become necessary for researchers to find effective treatment for oral infections. Natural products are an interesting alternative treatment that can control microbial growth without polluting the environment. These products are known to be cost-effective, relatively harmless and generally safer than synthetic agents (Yuan et al. 2016). Research for new antimicrobial agents obtained from plants has been of increased interest among researchers as alternative treatments can be used to replace the use of potentially damaging chemicals in the treatment or prevention of oral infections **.**Previous studies have reported that certain plants exhibit oral antimicrobial activity such as *Psidium guajava, Piper betle* (Fathilah et al. 2009) and *Camellia sinensis* (Anita et al. 2014), *etc.* The effective use of plant extracts in oral care products has also been studied and reported (Chandra et al. 2015).

Eupatorium stoechadosmum Hance is known as “San Pra Hom” in the Thai language. It is an aromatic plant with a strong spicy odor and belongs to the family Asteraceae. In traditional medicine, folk doctors have used the leaves of *E. stoechadosmum* to relieve pain and fever, to treat excessive sweating, degassing and to prevent bad breath in patients. Its roots have also been used to help woman maintain normal menstruation cycles and can be boiled with drinking water for the purposes of detoxification (Wuttidhamma, 2002). In Thai cuisine, the leaves of this plant are used in cooking as well as a seasoning. They can stimulate appetite and prevent certain unpleasant fishy and meaty smells. Previous studies on the *Eupatorium* species have reported the anti-inflammatory and cytotoxic activity of this plant against human cancer cell lines (Jiang et al. 2006). It has also been reported that some *Eupatorium* species) *E. areolare, E. arsenei, E. glabratum* and *E. pulchellum(* have exhibited antimicrobial activity against *Klebsiella pneumoniae* and *Staphylococcus aureus*, while also demonstrating efficacy against *Bacilus subtilis* and *Diplococcus pneumonia* (Garcia-Sanchez et al. 2015). However, there have only been a few studies on the antimicrobial activity of *E. stoechadosmum*, especially in relation to oral pathogens. Therefore, this study was conducted to assess the efficacy of *E. stoechadosmum* extract on inhibiting oral pathogens including the time-kill assay. Furthermore, the degree of antioxidant activity and cytotoxicity of *E. stoechadosmum* extract was studied to further determine its use as an alternative substance in the development of oral care products in the future.

MATERIALS AND METHODS

Chemicals and Culture media

The culture medium used in this experiment consisted of brain immersion broth (BHI), brain heart agar (BHA), saborodextrose broth (SDB), and saborodextrose agar. (SDA) were from Merck (Darmstadt, Germany). Hexane, Ethanol, and Ethyl Acetate were purchased from Carlo Erba (Bangkok, Thailand). Dimethyl sulfoxide (DMSO), 3-(4, 5-dimethyl thiazol-2- yl)-2, 5-diphenyl tetrazolium bromide (MTT) and 1,1-Diphenyl-2-picrylhydrazyl radical were sourced from Sigma-Aldrich (Saint Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) and Trypan Blue Stain were obtained from Gibco (New York, USA), while, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) *was* From Fluka Chemie AG (Buchs, Switzerland).

Plant Materials

The leaves of *E. stoechadosmum* were purchased from a local wholesale market in Chiang Mai Province located in northern Thailand between the months of February and March in 2018 . The botanical identification of the leaves was established at the Department of Biology, Chiang Mai University, Chiang Mai, Thailand. Fresh samples were washed before drying at 50°C in a hot air oven and ground to a fine powder.

Preparation of Plant Extract

Crude Ethanol

The dried powder sample was macerated with ethanol for 24 h at room temperature (Abubakar and Haque, 2020) at a ratio of 1:10 (g:mL). The mixture was then filtered with Whatman No.1 filter paper (Whatman, International Ltd., Middlesex, England) and the liquid phase was evaporated using a vacuum rotary evaporator (Buchi Rotavapor R-114, Switzerland). Subsequently, the crude ethanol extract (CE) was obtained and stored at 4°C in well-sealed and sterile bottles until used.

Fractionated Extract

Plant samples were subjected to solvent fractionations using 3 solvents with differing polarity values in ascending order (Abubakar and Haque, 2020). Samples were first macerated with hexane, followed by ethyl acetate and ethanol, respectively. The solvent was then evaporated to obtain hexane fractionated extract (F-Hexane), ethyl acetate fractionated extract (F-EtOAc) and ethanol fractionated extract (F-EtOH). All of extracts were stored at 4°C in well-sealed and sterile bottles until used.

Antimicrobial Activity of *E. stoechadosmum* Fractionated Extracts and Crude Extract

The oral microorganisms used in this study were *Streptococcus mutans* DMST41283 and *Candida albicans* DMST5815. The both of standard strains were kindly provided by the Department of Medical Science, Ministry

of Public Health, Nontaburi, Thailand. *S. mutans* was cultured in BHI at 37°C under anaerobic conditions (5% H_2, 5% CO_2 and 90% N_2) for 24-48 h, whereas *C. albicans* was cultured in SDB at 37°C under aerobic conditions for 24-48 h. Each suspension was prepared with a degree of turbidity equivalent to McFarland standard No. 0.5 with OD_{600} in a range of 0.08 - 0.100. Furthermore, the OD_{600} value was measured using a UV spectrophotometer Varian Cary IE (SpectralLab Scientific Inc., Canada). Antimicrobial activity of all plant extracts was performed using the agar disk diffusion method and the broth microdilution method, which determined minimum inhibition concentration (MIC), minimum bactericidal concentration (MBC), minimum fungicidal concentration (MFC) and time kill kinetic potential.

Disk Diffusion Method

The antimicrobial activity of *E. stoechadosmum* fractionated extracts and crude extract (CE) against *S. mutans* and *C. albicans* was determined using the method described by Bauer et al. (1996). Initial suspensions of *S. mutans* and *C. albicans* were adjusted to 0.5 McFarland standard with broth media before being tested. The suspensions were spread on the surface of BHI agar for *S. mutans* and SDB agar for *C. albicans* using a sterilize cotton swab. A 6 mm diameter sterile paper disk (Whatman International Ltd, Middlesex, England) was saturated with a 20 µL mixture of the plant extract in DMSO (200 mg/mL). The suspension was then dried at room temperature for 1 h and placed on the agar surface. Afterwards, these plates were incubated at 37 °C for 24 h under anaerobic and aerobic conditions for *S. mutans* and *C. albicans,* respectively. After being incubated, antimicrobial activity was determined by measuring the diameter of the clear zone in millimeters in order to identify the degree of inhibitory activity of the extracts. All experiments were done in triplicate. To establish controls, paper disks were soaked with Nystatin (20 mg/mL) and chlorhexidine (CHX, 1.2 mg/mL) as a positive control for antifungal and antibacterial activity tests, respectively. Accordingly, DMSO was then used as a negative control.

Broth Microdilution Method

E. stoechadosmum extracts were collected for determination of MIC using the broth microdilution method with some modifications (Sahin et al. 2003). The experiment was performed in a 96-well micro plate (Corning Incorporated, USA). The extracts were prepared by 2-fold serial dilution with broth media in the range from 100 mg/mL to 0.156 mg/mL. Broth media, Nystatin, Chlohexidine and DMSO were used as non-treated, positive and negative controls, respectively. The microplate was then incubated at 37°C for 24 h. After incubation, the MIC value was recorded as the lowest concentration of plant extract that could effectively inhibit the growth of *S. mutans* and *C. albicans* when the turbidity of the suspension was not altered. All dilutions of the MIC experiment were streaked on a BHA plate for *S. mutans* and an SDA plate for *C. albicans* and then incubated at 37°C for 24 h. After incubation, minimum concentrations of the plant extracts that exhibited no growth of *S. mutans* and *C. albicans* were recorded as minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC), respectively.

Time-Kill Assay

F-EtOH of *E. stoechadosmum* exhibited the highest antimicrobial activity against *S. mutans* and *C. albicans*, so it was chosen for killing time test. The potential of the extract was determined using the method described by Klepser et al. (1998). The initial suspension was prepared at a density of 1×10^6 CFU/mL. They were then seeded on the extract solution in 96 well plates at concentrations of 1, 2 and 4 times that of the MIC. The samples were incubated at 37 °C. At 0, 1, 2, 4, 6, 12 and 24 h, and volumes of 10 µL were then spread onto SDA and BHA for *C. albicans* and *S. mutans*, respectively. Plates were incubated at 37°C for 24 h to determine the amount of CFU/mL. The data were plotted as log CFU/mL versus time. Nystatin and CHX were used as a positive control for *C. albicans* and *S. mutans*, respectively. The experiments were performed in triplicate.

Determination of Antioxidant Activities

Antioxidant activity of the *E. stoechadosmum* extracts, including CE and the fractionated extracts, was evaluated by DPPH (2,2′-diphenyl-1-picrylhydrazyl) radical scavenging assay according to the method described by Brand- Williams et al. (1995) with some modifications. Subsequently, 100 µL of five serial concentrations of the extracts (31.3-500 µg/mL) and 100 µL of 0.2 mM DPPH in pure ethanol were put into each well of a 96-well microplate. Each of samples was thoroughly mixed and kept in the dark for 30 min at room temperature. The absorbance was then measured at 517 nm using a microplate reader (Spark M10, Tecan, Switzerland). Vitamin C, a natural antioxidant, and Trolox, a derivative of vitamin E, were used as standards. All determinations were performed in triplicate and the mean value of the experiments was determined and recorded. The scavenging activity was calculated based on the percentage of DPPH radical scavenging as follows: Antioxidant activity (%) = $[(A_{control} - A_{cample}) / A_{control}] \times 100$, where $A_{control}$ represents the absorbance of the DPPH solution and A_{sample} represents the absorbance of the extract with DPPH solution. The results were recorded as the extract concentrations required to inhibit 50% of the DPPH free radical (IC_{50}), which were determined from the plotted percentage inhibition curve versus the sample concentration. Accordingly, a lower IC_{50} value corresponds to a higher degree of antioxidant activity.

The ABTS (2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activity of the CE

and the fractionated extracts of *E. stoechadosmum* was determined using the method described by Arnao et al. (2001) with some modifications. ABTS radicals were freshly prepared by mixing 7 mM ABTS in water and 140 mM potassium persulfate. They were then stored in the dark at room temperature for 12 h. Free radical scavenging activity was investigated by mixing 50 µL of each sample at various concentrations (31.3-500 µg/mL) with 100 µL of ABTS working solution in each well of a 96-well microplate. The mixture was then kept at room temperature for 15 min to complete the reaction, and the decrease in absorbance was measured at 734 nm using a microplate reader. Trolox and Vitamin C were used as a standard. The ABTS radical cation scavenging activity was recorded at IC_{50}. The inhibition percentage of the ABTS radical was calculated using the following formula: $[(A_{control} - A_{cample}) / A_{control}] \times 100$, where $A_{control}$ represents the absorbance of ABTS solution and A_{sample} represents the absorbance of the sample with ABTS solution. All experiments were performed in triplicate.

Cytotoxicity Activity

The MTT (3-(4, 5-dimethyl thiazol-2- yl)-2, 5-diphenyl tetrazolium bromide) assay (Anto et al. 2003) was used to evaluate the cytotoxicity of *E. stoechadosmum* extracts. F-EtOH was used in this experiment as it exhibited the highest inhibitory activity against all tested pathogens. The cells used for testing were Human Gingival Fibroblast (HGF ATCC CRL 2014™). Cells were sub-cultured in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 incubator (Forma 310 incubator, USA) every 24 h. Viable cells were mixed with try pan blue dye and counted by a Cell Counter (Haemacy tometer; Hausser Scientific Horham, USA) after being adjusted to 2 x 10^4 cells/mL for experimental use.

In the experiment, 100 µL of viable cells were seeded in 96-well plates, and the plates were then incubated for 24 h in a 5% CO_2 incubator. Next, cell cultures were treated with 200 µL of *E. stoechadosmum* extracts at concentrations within a range of 0-1,000 µg/mL and incubated at 37°C for 24 h in a 5% CO_2 incubator. At the end of the incubation period, the medium of the reaction mixture was removed and cell survival was determined by MTT assay. Accordingly, 60 µL of the MTT solution (5 mg of MTT dissolved in PBS) was then added to each well. After incubation for 4 h, MTT solution was removed and 100 µL of DMSO was added to each well. The mixture was measured for absorbance at 570 nm using a plate reader (Tcan, Switzerland). The % relative viability of the cells was then calculated (A_{570} of treated sample/A_{570} of untreated sample) x 100. This experiment was performed in triplicate.

Statistical Analysis

Experiments were performed in three independent treatments. The results were analyzed and presented as means ± standard deviations (SD) using one-way ANOVA. A value of $p < 0.05$ was considered significant.

RESULTS

Preparation of Plant Extracts

The crude ethanol extract (CE) of *E. stoechadosmum* was obtained by ethanol maceration with a percentage yield of 19.44% w/w. The physical appearance of the CE was semi-solid, viscous, dark green in color and possessed a strongly spicy smell. The fractionated extraction of *E. stoechadosmum* was found to produce different yields. The results showed that F-EtOH gave the highest yield at 9.26% w/w, followed by F-EtOAc and F-Hexane at 6.88 and 4.36% w/w, respectively.

Antimicrobial Activity of *E. stoechadosmum* Fractionated Extracts and CE

The antimicrobial activity of CE and the fractionated extracts of *E. stoechadosmum* against *S. mutans* and *C. albicans* were observed using the agar disc diffusion method. Agar disk diffusion test demonstrated antimicrobial activity towards the inhibition zone size (relative ∅ in mm) as summarized in Table 1. CHX and Nystatin were used as positive control groups for fungal and bacterial quantification, respectively. Inhibitory activity was demonstrated against both *C. albicans* and *S. mutans*. DMSO was used as a negative control and did not display any inhibitory activity. The CE and fractionated extracts of *E. stoechadosmum* exhibited antimicrobial activity at different levels with zones of inhibition ranging from 9.35 ± 0.80 ***to*** 19.00 ± 0.30 mm. F-Hexane exhibited an inhibition zone of 10.80 ± 0.40 ***mm*** against *S. mutans*, whereas *C. albicans* indicated an inhibition zone of 9.35 ± 0.80 mm. F-EtOAc gave inhibition zones of 14.42 ± 0.40 and 12.50 ± 0.20 mm against *S. mutans* and *C. albicans*, respectively. Among the fractionated extracts, F-EtOH was found to be the most effective with inhibitory zones of 19.00 ± 0.30 and 17.90 ± 0.70 mm against *S. mutans* and *C. albicans*, respectively. Therefore, the results clearly show that F-EtOH exhibits inhibitory activity against both test pathogens with significantly higher than CE.

Table 1: Inhibition zones of CE and fractionated extracts of *E. stoechadosmum* against *S. mutans* and *C. albicans*.

Sample	Zone of inhibition (∅ in mm)*	
	S. mutans	***C. albicans***
Crude Ethanol (CE)	17.00 ± 0.80	16.70 ± 0.50
F-Hexane	10.80 ± 0.40	9.35 ± 0.80
F-EtOAc	14.42 ± 0.40	12.50 ± 0.20
F-EtOH	19.00 ± 0.30	17.90 ± 0.70
CHX	20.00 ± 0.00	-
Nystatin	-	18.10 ± 0.00
DMSO	0	0

* values are mean ± SD, 0 = no inhibition zone, - = not determined

MIC, MBC and MFC values of *E. stoechadosmum* extracts: MIC, MBC and MFC of the CE and fractionated extracts against *S. mutans* and *C. albicans* were determined to confirm the resulting of inhibition zones. The results are summarized in Table 2.

Table 2: MIC, MBC and MFC values of the CE and fractionated extracts of *E. stoechadosmum* against *S. mutans* and *C. albicans*.

Plant extract	*S. mutans*		*C. albicans*	
	MIC	MBC	MIC	MFC
Crude Ethanol	3.13	6.25	3.13	6.25
F-Hexane	6.25	12.50	6.25	12.50
F-EtOAc	6.25	12.50	6.25	12.50
F-EtOH	1.56	3.13	1.56	3.13
CHX	$< 6.2 \times 10^{-4}$	$< 6.2 \times 10^{-4}$	-	-
Nystatin	-	-	$< 9.8 \times 10^{-3}$	$< 9.8 \times 10^{-3}$

MIC: Minimum Inhibitory Concentration
MBC: Minimum Bactericidal Concentration
MFC: Minimum Bactericidal Concentration
- = not determined

CHX, a positive control for *S. mutans* exhibited the MIC and MBC values lower than 6.2×10^{-4} mg/mL. Nystatin, a positive control for *C. albicans* exhibited the MIC and MFC values lower than 9.8×10^{-3} mg/mL. Apparently, the MICs (mg/mL) of the extracts obtained from *E. stoechadosmum* on both strains, namely CE, F-Hexane, F-EtOAc and F-EtOH, were determined at 3.13, 6.25, 6.25 and 1.56 after 24 h at 37°C, respectively. MBCs and MFCs (mg/mL) derived from the effects of CE, F-Hexane, F-EtOAc and F-EtOH found to be the same, which were 3.12, 12.5, 25, 50 and 50 mg/mL, respectively. Among these extracts, F-EtOH proved to be most active followed by CE, while the same activity was observed in EtOAc and Hexane, respectively. Therefore, F-EtOH of *E. stoechadosmum* was selected for further study.

Time-Kill Assay

The F-EtOH of *E. stoechadosmum* was chosen for the kill-time test of *S. mutans* and *C. albicans* due to they showed the highest inhibitory activity against both of the test strain. The results were evident that the time required for killing of F-EtOH against *S. mutans* was determined to be dose-dependent, as shown in Figure 1.

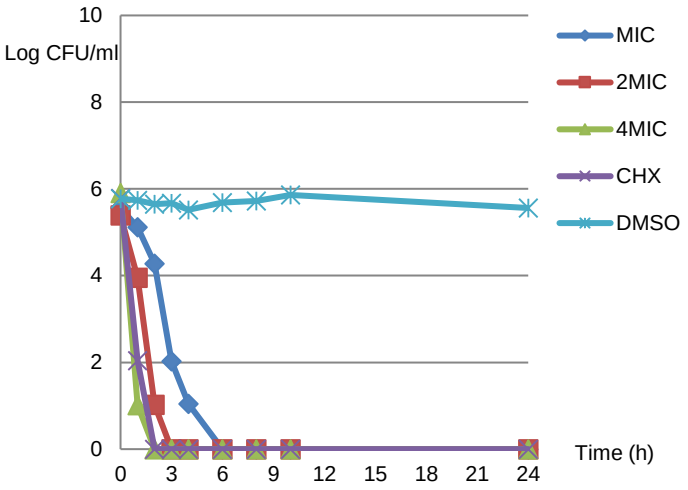

Figure 1: Time-kill assay against *S. mutans* of F-EtOH at concentrations of 1, 2 and 4 times of the MIC in comparison with CHX.

It was found that F-EtOH at concentrations of 1 times MIC (1.56 mg/mL), 2 times MIC (3.13 mg/mL) and 4 times MIC (6.25 mg/mL) indicated the efficacy of F-EtOH in killing *S. mutans* completely within 2 h. However, the strong degree of killing efficacy was evident when the concentration of F-EtOH was 4 times MIC (6.25 mg/mL) after being exposed to the inoculum. DMSO, a negative control for *S. mutans*, did not exhibit any inhibitory activity. CHX, a positive control, was able to kill *S. mutans* completely within 2 h. Apparently, the killing potential of F-EtOH against *S. mutans* was the same as CHX.

The killing time of F-EtOH against *C. albicans* was found to be dose dependent as is shown in Figure 2.

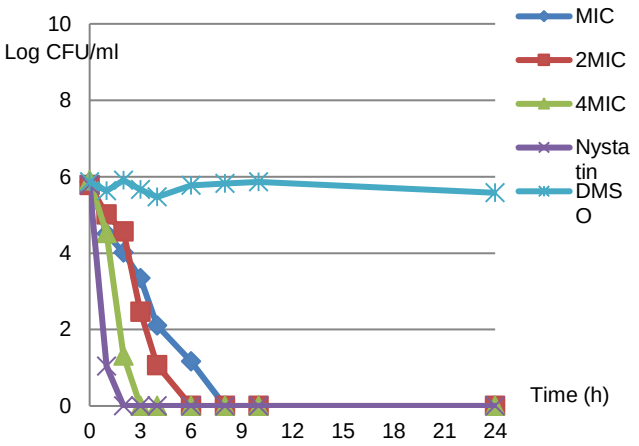

Figure 2: Time-kill assay against *C. albicans* of F-EtOH at concentrations of 1, 2 and 4 times of the MIC in comparison with Nystatin.

An increase in the concentrations of extracts from 1 times MIC (1.56 mg/ml) to 2 times MIC (3.13 mg/mL) and 4 times MIC (6.25 mg/mL) resulted in significantly higher

degrees of killing efficacy. At a concentration of 4 times MIC (6.25 mg/mL), F-EtOH completely kill *C. albicans* within 3 h. Accordingly, DMSO, a negative control, did not show any inhibitory activity. Nystatin, a positive control, could completely kill *C. mutans* within 2 h. Thus, the experimental results were clear that the killing time of F-EtOH against *C. albicans* was greater than Nystatin 1 h. Results were given in Table 1. 100 - 79 %

Antioxidant Activity

The radical scavenging activities of CE and the fractionated extracts of *E. stoechadosmum* using the methods of DPPH and ABTS radical scavenging activity assay. Results were given in Table 3.

Table 3: Antioxidant activity of the *E. stoechadosmum* extracts, including CE and The fractionated extracts, was determined using the DPPH and ABTS method.

	Antioxidant Activity	
	$IC_{50/DPPH}$ (mg/ml)	**$IC_{50/ABTS}$ (mg/ml)**
CE	0.393 ± 0.045	0.530 ± 0.007
F-Hexane	0.862 ± 0.110	1.036 ± 0.081
F-EtOAc	0.640 ± 0.080	0.711 ± 0.003
F-EtOH	0.104 ± 0.052	0.304 ± 0.042
Ascorbic acid	0.014 ± 0.001	0.029 ± 0.000
Trolox	0.058 ± 0.007	0.062 ± 0.011

values are mean ± SD

IC_{50} = extract concentrations required to inhibit 50% of free radicals

The scavenging effect of all extracts and the standard on the DPPH radical was expressed as IC_{50} values. The antioxidant activity of F-EtOH (IC_{50} value of 0.104 ± 0.052 mg/mL) exhibited the highest DPPH scavenging activity followed by CE (IC_{50} value of 0.393 ± 0.045 mg/mL) at 3.78 times, F-EtOAc (IC_{50} value of 0.640 ± 0.080 mg/mL) at 5.81 times and F-Hexane (IC_{50} value of 0.862 ± 0.110 mg/mL) at 8.29 times, respectively. However, the F-EtOH free radical scavenging efficacy was lower than that of Vitamin C (IC_{50} value of 0.014 ± 0.001 mg/mL) and Trolox (IC_{50} value of 0.058 ± 0.007 mg/mL) at 7.43 and 1.79 times, respectively.

For ABTS radical scavenging activity, the leaf extract (IC_{50} value of 0.304 ± 0.042 mg/mL) showed the highest scavenging activity followed by CE (IC_{50} value of 0.530 ± 0.007 mg/mL) at 1.74 times, F-EtOAc (IC_{50} value of 0.711 ± 0.003 mg/mL) at 2.34 times and F-Hexane (IC_{50} value of 1.036 ± 0.081 mg/mL) at 3.41 times, respectively. However, F-EtOH free radical scavenging efficacy was lower than that of Ascorbic acid (IC_{50} value of 0.029 ± 0.000 mg/mL) and Trolox (IC_{50} value of 0.062 ± 0.011mg/mL) at 10.48 and 4.90 times, respectively.

Cytotoxicity Activity

Percentages of human skin fibroblast viability after treatment with F- EtOH of *E. stoechadosmum* at concentrations in a range of 0-1,000 µg/mL are shown in Figure 3. The results indicate that F-EtOH retained a high degree of cell viability at 100-79%, and all concentrations were not significantly different from the control group. Therefore, the results of this experiment indicate that F-EtOH at concentrations within a range of 0-1,000 µg/mL was not found to exhibit cytotoxicity with cell viability greater than 79 %.

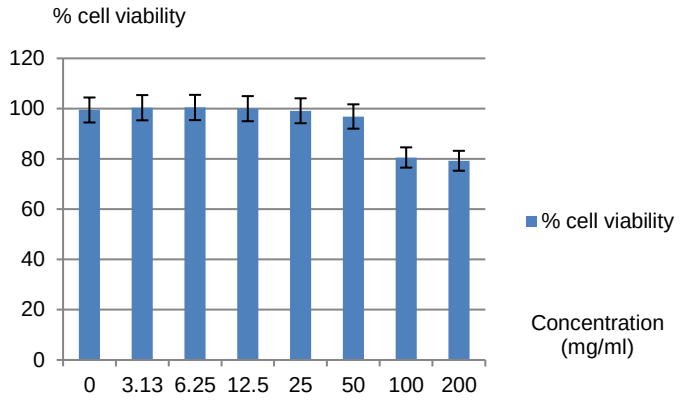

Figure 3: Percentages cell viability of human gingival fibroblast cells after treatment with varied concentrations of F- EtOH from *E. stoechadosmum*.

DISCUSSION

In the oral cavity, *S. mutans* and *C. albicans* are the main pathogens that cause dental caries, gingivitis and oral candidiasis. Mouthwash is a very effective substance in the prevention and treatment of oral infections and is widely used today. Most of mouthwashes contain chemical disinfectants, especially CHX has been found to have widely reported side effects. CHX is known to be highly effective in inhibiting microorganisms. Nystatin is highly effective for treatment oral candidiasis. However, nystatin, which has an unpleasant taste and associated with many negative effects, including nausea, vomiting, diarrhea, abdominal pain, anorexia as well as abdominal pain (Brautaset et al. 2011). In a study of the antimicrobial activity of *E. stoechadosmum* extract, DMSO was defined as a negative control by acting as an extract diluent without exhibiting any activity against *S. mutans* and *C. albicans*. This is due to the structure of DMSO consists of two polar and non-polar sections, which allows it to dissolve polar and non-polar substances and be transposed across hydrophobic barriers (Abreu et al. 2017). Therefore, DMSO was selected to dissolve the plant extracts in this research work. CHX and Nystatin were assigned as positive controls for *S. mutans* and *C. albicans,* respectively. CHX has antimicrobial activity due to its positively charged binds to negatively charged molecules of phospholipids in the inner membrane. This can lead to leakage of cellular components and cause cell death (Gurbani et al. 2015). Nystatin acts against fungi by

binding to sterols in the fungal cell membrane. The agent does not work with bacterial cells and mammals because they do not have sterols in their cell membranes. As a result, this binding action can cause changes in cell permeability, a loss of potassium and other cell components from fungal cells and can lead to cell lysis (Brautaset et al. 2011). A recent study was conducted on *E. stoechadosmum*, a widely cultivated plant in Asian countries. Its leaves have a strong pungent aroma and are routinely used by local people to prevent bad breath. There have been a number of reports of activity against various microorganisms, while oral antimicrobials have not been reported. 19.00 ± 0.30

The current study has reported on the inhibitory effect of *E. stoechadosmum* extracts against both test strains including oral pathogenic bacteria *S. mutans* DMST 41283 and oral pathogenic fungi *C. albicans* DMST 5815. The antibacterial and antifungal activities were investigated using the disk diffusion method and the broth microdilution method. F-EtOH was found to be the most effective with inhibitory zones of 19.00 ± 0.30 and 17.90 ± 0.70 mm against *S. mutans* and *C. albicans*, respectively. In comparison, it was shown that the CE has potential to inhibit *S. mutans* and *C. albicans*. However, the inhibition zones of CE against both tested oral pathogens were significantly less than that of F-EtOH. The inhibition efficacy of F-EtOH against both pathogens was also confirmed by MIC values and MBC values for *S. mutans* and MFC values for *C. albicans*. This outcome was also confirmed by the results of the time-kill test. This may have been due to the manner in which their chemical constituents display different antimicrobial properties. It has been reported that thymohydroquinone dimethyl ether (73.6%), caryophyllene (8.9%) and selina-4,11-diene (11%) were found to be the main constituent in essential oils derived from *E. stoechadosmum* (Dung et al. 1991). In addition, *E. stoechadosmum* was found to be active in the screening of oral bacterial that produce VSCs causing halitosis (Li et al. 2010). Meanwhile, coumarin and thymol derivatives obtained from *E. stoechadosmum* could inhibit *C. albicans* (Sobrinho et al. 2017; Yasameen et al. 2017). Additionally, Ahmad and colleagues reported that alcohol was a more effective solvent for the extraction of antimicrobial active compounds when compared to water and hexane (Ahmad and Beg, 2001).

Antioxidant activity in this study was determined using DPPH and ABTS methods. The DPPH and ABTS scavenging activity values obtained in this study are used as an index to assess the effects of antioxidants that can be applied in the prevention and elimination of free radicals during cell activity. Ascorbic acid, a natural antioxidant, and Trolox, a derivative of vitamin E, were used as standards. The results of this study indicated that F-EtOH showed the greatest efficacy in DPPH with IC_{50} (mg/mL) values of 0.104 ± 0.052 when compared to other extracts of *E. stoechadosmum*, but these values were lower than for Vitamin C (IC_{50} value of 0.014 ± 0.001 mg/mL) and Trolox (IC_{50} value of 0.058 ± 0.007 mg/mL) at 7.43 and 1.79 times, respectively. Likewise, F-EtOH was found to be the most effective in scavenging

ABTS radicals with an IC_{50} value of 0.052 ± 0.104 mg/mL, but this was lower than the standard values for Vitamin C (IC_{50} value of 0.014 ± 0.001 mg/mL) and Trolox (IC_{50} value of 0.058 ± 0.007 mg/mL) at 7.43 and 1.79 times, respectively. The antioxidant activity of natural extracts was found to be correlated with its constituents and was mainly related to the total phenols and flavonoids contained in the extracts. In this study, F-EtOH displayed better antioxidant activity than the other extracts. According to the outcomes of previous studies (Pham et al. 2018), o-coumaric acid isolated from *E. stoechadosmum* was reported to possess strong antibacterial and antioxidant properties. A thymol derivative obtained from *E. stoechadosmum* that was extracted with ethanol has been confirmed to exhibit high antioxidant activity (Phan et al. 2019). Phenolics, such as thymol, carvacrol and thymoquinone, exhibited high antioxidant and health-promoting activities. Phytochemical reports have indicated the occurrence of monoterpenoids, sesquiterpenoids, triterpenoids, thymol derivatives, flavonoids and acetophenone obtained from *E. stoechadosmum.* Importantly, these compounds are recognized as potent antioxidants (Trang et al. 1993).

MTT assay was used to evaluate the degree of cytotoxicity of F-EtOH against normal Human gingival fibroblast cells. In general, natural extracts are known to exhibit a lower degree of cytotoxicity when compared to synthetic chemical compounds. Most of the previous studies conducted on the cytotoxicity of *E. stoechadosmum* have focused on its anticancer activity against cancer cells, while the cytotoxicity on human gingival fibroblasts has not yet been fully studied. The cytotoxicity assays conducted in this study have clearly shown that F-EtOH maintained high cell viability (>79 %) up to a concentration of 1000 µg/mL.

CONCLUSION

In conclusion, the results of this study demonstrated that F-EtOH derived from *E. stoechadosmum* exhibited strong antimicrobial and antioxidant activity, as well as low cytotoxicity. Based on these results, F-EtOH can be used as an ingredient in natural oral care products, particularly mouthwash, to effectively treat infections of *S. mutans* and *C. albicans.*

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest

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AUTHOR CONTRIBUTIONS

All authors participated in the design of the experiment, conducted the experiment, analyzed the experimental results, reviewed and edited the manuscript. All authors have read and agree to publish the manuscript.

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