

Antioxidant, Anticancer and Antimicrobial Activities of Methanolic Extracts from *Encisanthellum pulchrum* (King) Heusden

(Aktiviti Antioksidan, Antikanser dan Antimikrobia Ekstrak Kasar *Encisanthellum pulchrum* (King) Heusden)

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ABSTRACT

Biological activities of crude methanolic extracts from leaves, barks, twigs and roots of Encisanthellum pulchrum were investigated in four bioassays. The antioxidant, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay showed that bark and twig extracts showed high inhibitory activity with 60 and 56% inhibition at 1 mg/mL and IC₅₀ values of 0.43 ± 0.04 and 0.64 ± 0.05 mg/mL, respectively. The bark and root extracts showed greater reducing power (FRAP) than several standard drugs used in the bioassay. Methanolic extracts of leaves, twigs and roots displayed strong cytotoxicity to breast cancer cell line (MCF-7), myelomonocytic leukaemia cell line (WEHI-3) and ovarian cancer cell line (CAOV-3); the IC₅₀ of the leaf extract were 7.8 ± 0.85 µg/mL (MCF-7) and 9.0 ± 0.13 µg/mL (WEHI-3), while those for the twig and root extracts were 13.9 ± 0.35 and 7.3 ± 0.98 µg/mL (CAOV-3), respectively. In the antimicrobial assays, the extracts were tested against ten bacterial strains and two fungal strains. Bark and twig extracts displayed high inhibitory activity to Bacillus subtilis with 13.3 ± 0.57 and 12.0 ± 0.01 mm inhibition, respectively. In addition, the twig extract displayed better minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) compared with the bark extract (MIC 0.5 and 1.0 mg/mL, MBC 1.0 and 2.0 mg/mL, respectively). For antifungal activity, all extracts showed inhibition on Candida albicans but not on Aspergillus niger. The obtained results suggested that this plant may possibly contain bioactive compounds in the active extracts.

Keywords: Anticancer; antimicrobial; antioxidant; Encisanthellum pulchrum; methanolic extracts

ABSTRAK

Aktiviti biologi Encisanthellum pulchrum bagi ekstrak kasar metanol daun, kulit, batang dan akar telah dikaji terhadap empat bioasai. Asai skaveng antioksidan, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) mendedahkan bahawa ekstrak kulit dan batang menunjukkan aktiviti perencatan yang tinggi dengan perencatan sebanyak 60 dan 56% pada kepekatan 1 mg/mL, manakala nilai IC₅₀ masing-masing adalah 0.43 ± 0.04 dan 0.64 ± 0.05 mg/mL. Ekstrak kulit dan akar menunjukkan kuasa pengurangan yang lebih besar (FRAP) berbanding dadah kawalan yang digunakan dalam bioasai ini. Ekstrak metanol daun, batang dan akar memaparkan sitotoksiti yang kuat terhadap titisan sel kanser payudara (MCF-7), leukimia milomonositik (WEHI-3) dan kanser ovari (CAOV-3); nilai IC₅₀ bagi ekstrak daun 7.8 ± 0.85 µg/mL (MCF-7), manakala ekstrak batang dan akar masing-masing adalah 13.9 ± 0.35 dan 7.3 ± 0.98 µg/mL (CAOV-3). Bagi asai antimikrob, ekstrak diuji terhadap sepuluh strain bakteria dan dua strain fungus. Ekstrak kulit dan batang memaparkan aktiviti perencatan yang tinggi terhadap bakteria bacillus subtilis dengan perencatan masing-masing sebanyak 13.3 ± 0.57 dan 12.0 ± 0.01 mm. Bacaan bagi kepekatan perencatan minimum (MIC) dan kepekatan bakterisidal minimum (MBC) bagi kedua-dua ekstrak menunjukkan ekstrak batang memberikan bacaan MIC dan MBC yang lebih baik berbanding ekstrak kulit dengan bacaan masing-masing sebanyak (MIC 0.5 dan 1.0 mg/mL, MBC 1.0 dan 2.0 mg/mL). Bagi aktiviti antifungus, semua ekstrak memberikan perencatan terhadap Candida albicans dan tidak pada Aspergillus niger. Keputusan yang diperoleh mencadangkan bahawa tumbuhan ini berkemungkinan mempunyai sebatian bioaktif daripada ekstrak aktif.

Kata kunci: Antikanser; antimikrob; antioksidan; ekstrak metanol; Encisanthellum pulchrum

INTRODUCTION

Natural products have been recognized as an important tool in the drug discovery process throughout this century (Agosta 1997; Sthrol 2000). Many studies have been conducted especially on plants in order to identify chemical compounds that can act as remedies for various diseases. In fact, plants have been reported to be used worldwide

for medicinal purposes (Duke 2000). However, the search for biologically active compounds requires a bioassay procedure to detect a certain type of biological activity in the corresponding crude extract (Hostettmann et al. 1997). The development of biological activity studies on the plant extracts and compounds in Malaysia actively began in the early 90's (Jantan 2008). Some specific

bioassay method involving *in vitro* systems have been used to study biological activities such as anticancer, antiviral and antiparasitic (Jantan 2008). Several reports have been published regarding the study of the biological activity of extracts and compounds from local plants such as antimalarial, antimicrobial, antioxidant, anti-inflammatory and cytotoxic activities of *Garcinia atroviridis*, *Sandoricum koetjape* and *Hedyotis* species (Ahmad et al. 2005; Mackeen et al. 2002; Mat Ali et al. 2004; Nik Rahman et al. 1999). Therefore, plant derived-bioactive compounds have received considerable attention due to their therapeutic potential as antimicrobial, anti-inflammatory, anticancer and antioxidant agents (Rathee et al. 2009).

Enicosanthellum pulchrum (King) Heusden also known as family of 'mempisang' is a species from Annonaceae family (Burkill 1966). It was first discovered in the Coteau area at the border of Thailand and Malaysia. It is a coniferous tree that can grow to about 3-5 m tall (David et al. 1989). This plant is a highland plant which is confined to mountain forests at an altitude of 1200-1500 m (Ng et al. 1990). Phytochemical studies of this plant have shown several isoquinoline alkaloids such as (-)-asimilobine, (-)-anonaine, (-)-norliridine, liriodenine and (-)-scoulerine (Lavault et al. 1990) as well as azaantraquinone alkaloid such as cleistopholine (Nordin et al. 2012). However, to the best of our knowledge, only one biological activity has been reported so far for this plant which is anti-platelet activating factor (Nordin et al. 2012). In the current study, we investigated the presence of bioactive compounds from each part of *E. pulchrum* and its antioxidant, anticancer and antimicrobial potential in methanolic extracts.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

2,2-Diphenyl-1-picrylhydrazyl (DPPH), fetal bovine serum (FBS), sodium nitrite, quercetin, trolox, ascorbic acid, sodium acetic acid and iron chloride were purchased from Sigma-Aldrich. Dimethylsulfoxide (DMSO), acetic acid glacial, hydrochloric acid, methanol and ethanol were purchased from Merck Co. (Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Invitrogen (Carlsbad, USA). RPMI-1640 medium (pH7.4) from Nacalai tesque (Japan) and Trypsin-EDTA 10X from Biowest (USA).

CELL LINE AND PATHOGEN

Eleven cell lines including normal hepatic cells (WRL-68), breast cancer cells (MCF-7 and MDA-MB231), human hepatocellular carcinoma cells (HepG2), colon adenocarcinoma cells (HT-29), normal colon cells (CCD-841), ovarian cancer cells (CAOV-3, SKOV-3), prostate adenocarcinoma cells (PC3), myelomonocytic leukaemia cells (WEHI-3B) and human promyelocytic leukaemia cells (HL-60) were obtained from American Type Culture Collection (ATCC). Twelve pathogens in total,

including ten bacteria strains, *Bacillus cereus* (B43), *Bacillus subtilis* (B145), *Enterobacter cloacae* (UMMC), *Staphylococcus aureus* (S1434), *Salmonella typhimurium* (S1211), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (UMMC), *Staphylococcus aureus* (ATCC 25923), *Pasteurella multocida* (UMMC) and *Staphylococcus epidermidis* (UMMC) as well as two fungal strains, *Candida albicans* (C523) and *Aspergillus niger* (A121) were used for antimicrobial assay.

PLANT PREPARATION

The plant *E. pulchrum* was collected from Cameron Highlands Montane Forest, Pahang. The specimen was identified by a botanist, the late Prof. Dr. Kamaruddin Mat Salleh from the Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The voucher specimen (SM769) was deposited at the Herbarium of the Botany Department, UKM. Each plant including leaves, barks, twigs and roots were air-dried and ground to 40-60 mesh size before extracted with methanol by maceration technique. Evaporation of the solvents using rotary evaporator gave four crude methanolic extracts.

DPPH RADICAL-SCAVENGING ACTIVITY

The DPPH assay was performed according to the modified method by Orhan et al. (2007) and Brem et al. (2004). Briefly, 100 μ L of DMSO was added into each 96 wells. Then, 100 μ L (1 mg/mL) of standard compounds or samples (1 mg/mL) were added into the first row. Subsequently, 10 μ L (2.5 mg/mL) of stable DPPH free radical in DMSO were added to each well in a 96-well plate. Ascorbic acid (vitamin C) and quercetin were used as standard drug whereas blank solvent DMSO as the negative control. The absorbance was read at 517 nm using microplate absorbance reader (Infinite M200PRO) after 20 min of incubation at room temperature.

FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

Determination of the total antioxidant activity (FRAP assay) was using a modified method of Benzie and Strain (1999). The stock solutions included 300 mM acetate buffer (3.1g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 mL $C_2H_4O_2$), pH3.6, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution (FRAP reagent) was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3 \cdot 6H_2O$. 10 μ L of samples (1 mg/mL) and 300 μ L FRAP reagent were added in each wells. Readings of the coloured product (ferrous tripyridyltriazine complex) were taken at 593 nm.

CYTOTOXICITY ASSAY (MTT ASSAY)

All cells were grown in RPMI 1640 medium and maintained in 37°C incubator with 5% CO_2 saturation. Different cell types were used to determine the cytotoxic effects of *E. pulchrum* extracts using the MTT assay. Confluent cells were centrifuged at 1800 rpm for 5 min and concentration

of cells was adjusted to 1×10^6 cells/mL. To measure cell viability, 100 μ L of cells were seeded in a 96-well plate and incubated for 24 h at 37°C. Cells were then treated with the extracts and incubated for 24 h. After 24 h, MTT solution (5 mg/mL) was added in each well for 3 h. Absorbance was measured at 570 nm using an ELISA microplate reader. The results were shown as a percentage of control giving percentage cell viability after 24 h exposure to test agents (Cheah et al. 2011). Several positive controls such as tamoxifen, paclitaxel, cisplatin and vinblastin were used in the experiment.

DISC DIFFUSION ASSAY

For bacterial disc diffusion assay, petri plates (9 cm) were prepared with 20 mL of a base layer of Nutrient Agar (DIFCO, Becton Dickinson, USA) whereas for the fungal disc diffusion assay, Potato dextrose agar (PDA) was used. Each petri plate was inoculated with 15 μ L of each bacterial/fungal suspension (10^6 CFU/mL). After being dried in a sterile hood, 6 mm diameter discs soaked with 10 μ L of the extracts from *E. pulchrum*. Discs containing standards antibiotics were used as positive control. The plates were incubated for 24 h at 37°C. The diameters of the inhibition zones were evaluated in millimeters. All tests were performed in triplicate and the bacterial/fungal activity was expressed as the mean of inhibition diameters (mm).

DETERMINATION OF MIC AND MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antibiotics and plant extracts were determined using the standard broth micro dilution method as described by Eloff (1998) using test bacteria that have been standardized to OD600 nm. Extracts

were dissolved in 100% DMSO to obtain a concentration of 1 mg/mL. Using a 96-well microtiter plate, nutrient broth, bacteria and extracts were dispensed into every well and labelled appropriately. Plates were covered and incubated at 37°C for 24 h. Control wells were loaded with nutrient broth and extracts with no bacteria added (wells 12 A-H), broth and bacteria with no extract (wells 11 A-H). Tecan micro plate reader (Infinite M200PRO) was used to quantify the optical density of the reactants in each well. The MBC of active extracts were determined by streaking on nutrient agar plates from wells that showed growth inhibition. The concentration of extract in the wells where there was no growth on plate was considered as the MBC.

STATISTICAL ANALYSIS

All values were reported as mean \pm standard deviation (SD) of three replicates. Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test to determine statistical significance. GraphPad Prism 5 software (GraphPad Software Inc. San Diego, CA) was used for all statistical analyses as well as for IC₅₀ values.

RESULTS AND DISCUSSION

EVALUATION OF ANTIOXIDANT ACTIVITY

Methanolic extracts of leaves, barks, twigs and roots of *E. pulchrum* were investigated for antioxidant activity. The results of ferric reducing power are presented in Figure 1. In this study, the bark extract showed strong ability in reducing Fe³⁺ to Fe²⁺ with 1491.4 ± 0.03 μ mol/L followed by root and twig extracts (1078.1 ± 0.03 and 941.1 ± 0.03 μ mol/L, respectively). Meanwhile, the leaves extract

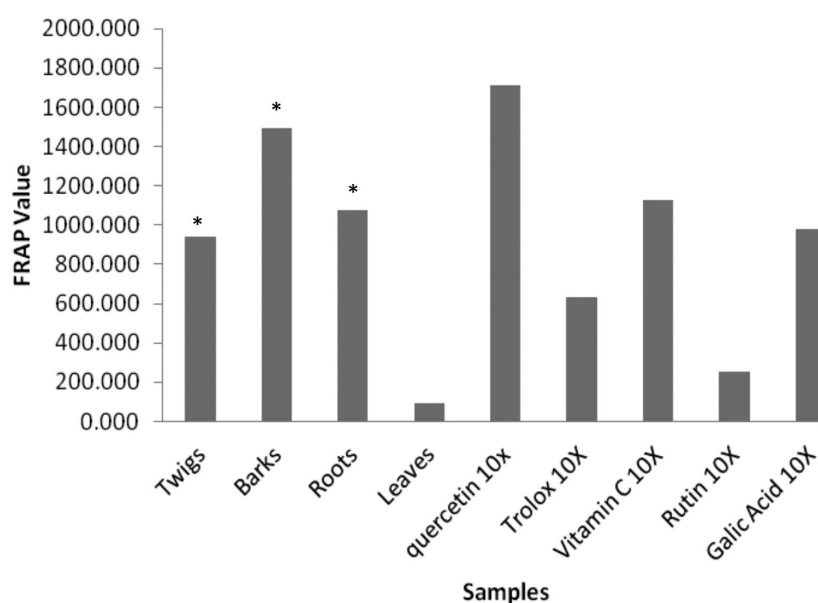


FIGURE 1. Reducing power activity of the crude methanol extract of *E. pulchrum*. * $p < 0.05$ indicates no significant difference compared to the positive control

exhibited the lowest FRAP value was $91.6 \pm 0.01 \mu\text{mol/L}$. The radical scavenging activity using DPPH was tested for all extracts (Figure 2). Inhibitory activity was calculated in the following order: barks extract ($60.4 \pm 0.02\%$), twigs extract ($55.8 \pm 0.05\%$), roots extract ($20.2 \pm 0.01\%$) and leaves extract ($7.34 \pm 0.04\%$). Percentage inhibition of more than 50% was calculated for their IC_{50} values (Table 1). The IC_{50} results showed that barks and twigs exhibited weak of free radical scavenging activities of 430.0 ± 0.04 and $640.0 \pm 0.05 \mu\text{g/mL}$ compared with ascorbic acid ($70.0 \pm 0.23 \mu\text{g/mL}$) as a positive control. The antioxidant results obtained from the *E. pulchrum* extracts may be due to the presence of phenolic compounds in the extract. Phenolic compounds have been known as antioxidant and scavenging agents against free radicals associated with oxidative damage (Ferguson et al. 2006). This was supported by previous studies which demonstrated the strong antioxidant activity of phenolic compounds against diseases associated with oxidative stress. Phenolic compounds such as (+)-catechin and (-)-epicatechin were found to be the most active with IC_{50} values of 4.16 and $4.67 \mu\text{M}$ for DPPH and 190 and $170 \mu\text{M}$ for nitric oxide scavenging activities (Kirmizibekmez et al. 2009). A recent study of phenolic compounds and antioxidant activity reported that isoflavone aglycones (genistein, daidzein and glycitein), isoflavone β -glucosides (genistin, daidzin and glycitin) and phenolic acids also contribute to the antioxidant activity (Lee et al. 2013). Thus, the presence of phenolic compounds as the best electron donors can eliminate the radical chain reaction by converting free radicals to more stable products (Mbaebie et al. 2012).

EVALUATION OF ANTICANCER ACTIVITY

The cytotoxic activity was determined by a tetrazolium (MTT) assay. The results of all extracts are presented in

TABLE 1. The IC_{50} values of DPPH assay of active extracts of *E. pulchrum*. All values represent means \pm SD of three replicates

Samples	IC_{50} ($\mu\text{g/mL}$)
Ascorbic acid	70.0 ± 0.23
Bark	430.0 ± 0.04
Twig	640.0 ± 0.05

Table 2. The cell survival decreased in a dose-dependent manner. Three extracts of leaves, twigs and roots exhibited very low IC_{50} values on breast cancer cell (MCF-7), ovarian cancer cell (CAOV-3) and myelomonocytic leukaemia cell lines (WEHI-3), confirming these extracts had the highest toxicity. Leaves extracts were shown to be more active on MCF-7 cell line (IC_{50} : $7.8 \pm 0.85 \mu\text{g/mL}$) and WEHI-3 cell line (IC_{50} : $9.0 \pm 0.13 \mu\text{g/mL}$) while twigs and roots were active on CAOV-3 cell line (IC_{50} : 13.8 ± 0.35 and $7.3 \pm 0.98 \mu\text{g/mL}$). Methanolic extracts of *E. pulchrum* did not show any toxicity effects on normal hepatic cells (WRL-68) up to $100 \mu\text{g/mL}$. Findings from this study showed extracts from leaves, twigs and roots may contain bioactive compounds that demonstrate their potential to be an anticancer drug. Based on cytotoxicity screening results on all cancer cell lines tested, these extracts exhibited very low IC_{50} on breast cancer cells and myelomonocytic leukaemia cells (leaves) as well as ovarian cancer cells (twigs and roots). Both type of cancer except myelomonocytic leukaemia are the most common cancer in women. Thus, this plant has the potential to treat cancer that effects women. Meanwhile, phytochemical studies carried out by Lavault et al. (1990) and Nordin et al. (2012) found that chemical compounds present in *E. pulchrum* are alkaloids. This group of compound has been shown to be highly active against several cancer cell (Lu et al. 2012). In fact, alkaloids are among the most important active components in natural

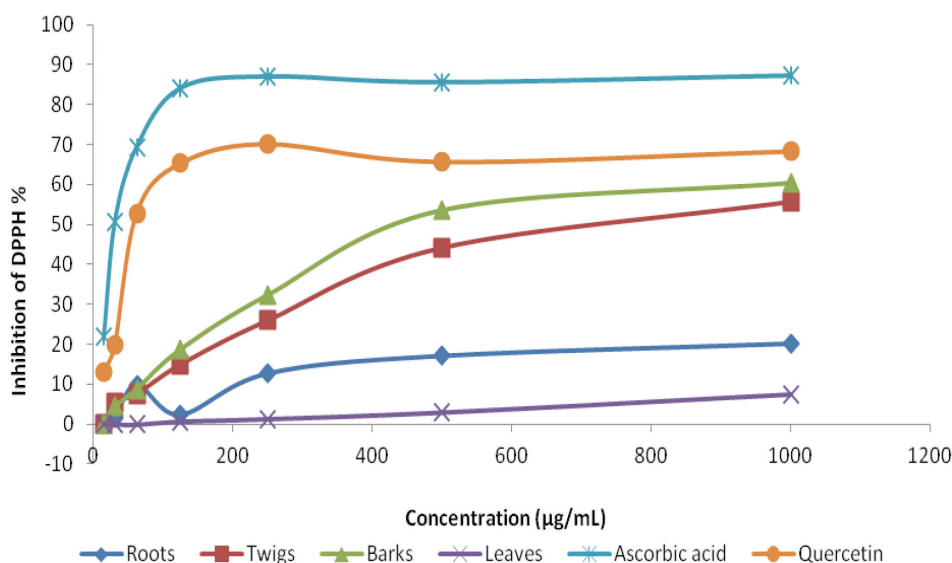


FIGURE 2. Effect of different extracts of *E. pulchrum* on DPPH scavenging activity. Data represent means \pm SD of three replicates

TABLE 2. Effect of methanolic extracts of *E. pulchrum* on several cell lines

IC50 µg/mL	MCF-7	MDA-MB231	HEPG2	HT-29	CCD841	SKOV-3	CAOV-3	PC3	WEHI-3B	HL-60	WRL-68
Leaves	7.8±0.85	>50	>50	49.5	>50	>50	>50	>50	9.0±0.13	>50	94.5
Twigs	>50	44.6	>50	>50	>50	>50	13.9±0.35	>50	>50	>50	>100
Barks	43.2±1.69	37.2±9.05	>50	48.4	>50	>50	37.2±6.15	>50	31.0±0.11	36.0±0.01	99
Roots	>50	46.8	>50	>50	>50	>50	7.3±0.98	47.1	>50	>50	99.5
Tamoxifen	1.01±1.21	6.68±1.34	-	30.3±2.1	-	-	-	-	-	-	-
Paclitaxel	-	-	-	-	33.2±1.3	4.3±0.3	<0.79±0.1	0.3±0.1	-	-	26.8±1.13
Vinblastin	-	-	-	-	-	-	-	-	2.0±1.2	1.8±0.1	-
Cisplatin	-	-	25.6±1.3	-	-	-	-	-	-	-	-

*All values represent means ± SD of three replicates

herbs and some of these compounds have already been successfully developed into chemotherapeutic drugs, such as vinblastine, which interacts with tubulin (Li et al. 2007), camptothecin (CPT) and a famous topoisomerase I (TopI) inhibitor (Huang et al. 2007). Several alkaloids isolated from this plant also showed anticancer activity in human breast and lung cancer cell lines including asimilobine, annonaine and liriodenine (Yeh et al. 2011). Among these alkaloids, liriodenine exhibited highly active anticancer activity against both cancer cell line (Yeh et al. 2011). Therefore, the bioactive compounds which consist of alkaloids may contribute to the anticancer activity.

EVALUATION OF ANTIMICROBIAL ACTIVITY

The inhibition zone produced by *E. pulchrum* extracts against ten bacteria and two fungi can be seen in Tables 3 and 5. Bark and twig extracts displayed high inhibitory zones against *B. subtilis* at 13.33 ± 0.57 and 12.0 ± 0.01 mm, respectively. However, none of the extracts exhibit activity against *E. cloacae* and *P. aeruginosa*. The two extracts showing highest inhibition zone were subjected to MIC and MBC tests against *B. subtilis*. The MIC and MBC values of twigs extract were lower than that of the bark extract (MIC 0.5 and 1.0 mg/mL, MBC 1.0 and 2.0 mg/mL,

respectively) (Table 4). This could be due to the presence of specific groups in the compounds of the extracts. Meanwhile, the results of antifungal activity exhibited only leaves and twig extracts gave the same inhibition value (9.0 mm) against *C. albicans*. Based on the results, all extracts showed activity against all tested bacteria and fungi except *E. cloacae*, *P. aeruginosa* and *A. niger*. It is interesting to note that conventional drugs in use are more active against Gram-positive than Gram-negative bacterial strains (Mewari & Kumar 2008). Lin et al. (1999) also reported that the plant extracts are usually more active against Gram-positive than Gram-negative bacteria. This may be due to the higher resistance of Gram-negative bacteria against antibiotics because of their impenetrable cell wall. In addition, lipopolysaccharide (LPS) content possessed by Gram-negative bacteria also contribute to its resistivity. There are many studies that showed the significant effect of plant extracts against Gram-positive compared with Gram-negative bacteria, such as antibacterial activity of extracts from plants in central Argentina (Joray et al. 2011) and evaluation of various crude extracts of *Zingiber ficinale* rhizome for potential antibacterial activity (Kaushik & Goyal 2011). Our findings also exhibited the extract of *E. pulchrum* were more active towards Gram-positive bacteria (*B. cereus*, *B. subtilis* and *S. aureus*) than Gram-negative

TABLE 3. Inhibition diameter of *E. pulchrum* methanolic extracts against ten bacterial strains

Bacterial strains / Extracts	Inhibition diameter (mm)				
	Leaves	Barks	Twigs	Roots	Streptomycin
<i>B. cereus</i>	7±0	7±0	7.33±0.57	NI	35.0±0
<i>B. subtilis</i>	7±0	13.33±0.57	12±0	NI	35.0±0
<i>E. cloacae</i>	NI	NI	NI	NI	22.0±0
<i>S. aureus</i> (S1434)	8.33±0.57	8.33±0.57	8±0	NI	20.0±0
<i>S. typhimurium</i>	8±0	8.66±0.57	8.66±0.57	NI	16.0±0
<i>P. aeruginosa</i>	NI	NI	NI	NI	-
<i>K. pneumoniae</i>	8±0	7.66±0.57	7.66±0.57	8±0	24±0
<i>S. aureus</i> (27853)	7±0	NI	NI	NI	20.33±0
<i>P. multocida</i>	9±0	8.6±0.57	9±0	NI	24±0
<i>S. epidermidis</i>	7±0	7.3±0.57	7.6±0.57	NI	22.0±0

* All values represent means ± SD of three replicates. NI – no inhibition observed

TABLE 4. The MIC and MBC values of active extracts of *E. pulchrum* against *B. subtilis*

Samples	Concentration (mg/mL)	
	MIC	MBC
Barks	1.0	2.0
Twigs	0.5	1.0
Streptomycin	9×10^{-4}	9×10^{-4}

TABLE 5. Inhibition diameter of *E. pulchrum* methanolic extracts against two fungal strains. All values represent means \pm SD of three replicates

Samples/ Fungal strain	Diameter (mm)	
	<i>C. albicans</i>	<i>A. niger</i>
Leaves	9.0 \pm 0	NI
Twigs	7.0 \pm 0	NI
Barks	9.0 \pm 0	NI
Roots	7.0 \pm 0	NI
Nystatin	23.33 \pm 1.15	-

*NI- no inhibition observed

bacteria (*E. cloacae*, *S. typhimurium*, *P. aeruginosa*, *K. pneumoniae*, *P. multocida* and *S. epidermidis*).

CONCLUSION

The results indicated that leaves, twig and root extracts of *E. pulchrum* may contain promising therapeutic anticancer but not antioxidant and antimicrobial properties. At present, the isolation study is being conducted to purify the compounds from the active extracts.

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