

## ***Eupatorium ayapana*, a natural source of anti-biofilm, anti-inflammatory and anti-oxidant agents**

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### **Abstract**

*Eupatorium ayapana* is one of the most important plants used in herbal medicine. The plant is considered to be a therapeutic agent for the treatment of various diseases. The objective of this research was to evaluate anti-biofilm, anti-inflammatory, and anti-oxidant activities of the plant extracts. *E. ayapana* leaves were extracted with hexane, CH<sub>2</sub>Cl<sub>2</sub>, and EtOAc solvents and the extracts were further utilized in biological assays. The results demonstrated that all of the extracts exhibited pronounced anti-biofilm formation against *Escherichia coli* in a dose-dependent manner. *E. coli* biofilm formation was inhibited more than 80% after treatment with the CH<sub>2</sub>Cl<sub>2</sub> and EtOAc extracts (1,024 µg/ml) compared with untreated cells. In addition, the microorganism produced biofilm 40% less after treatment with 1,024 µg/ml hexane extract. Moreover, the established bacterial biofilm also decreased after treatment with 1,024 µg/ml of all the extracts. For anti-oxidant activity, the EtOAc extract exhibited excellent activity against DPPH radicals with the half inhibition concentration (IC<sub>50</sub>) value at 22.7 µg/ml, which is very closed to that of BHT (24.3 µg/ml). In addition, the CH<sub>2</sub>Cl<sub>2</sub> and EtOAc extracts exhibited good anti-inflammatory activity against nitric oxide with IC<sub>50</sub> values at 65.7 and 66.9 µg/ml, respectively. The differences in biological activities of the extracts of *E. ayapana* can be used as novel anti-biofilm, anti-inflammatory, and anti-oxidant agents.

**Keywords:** *Eupatorium ayapana*, anti-biofilm formation, anti-inflammatory, anti-oxidant activity

### **บทคัดย่อ**

ต้นพริกไทยเป็นหนึ่งในพืชที่มีความสำคัญทางด้านยาสมุนไพร โดยพืชชนิดนี้ใช้ในการรักษาโรคได้หลากหลายชนิด วัตถุประสงค์ในการศึกษาครั้งนี้เพื่อทดสอบฤทธิ์ต้านไบโอฟิล์ม ฤทธิ์ต้านการอักเสบและฤทธิ์ต้านอนุมูลอิสระ โดยนำใบของต้นพริกไทยมาสกัดด้วยตัวทำละลายอินทรีย์ ได้แก่ เฮกเซน ไคลอโรฟอร์ม และเอทิลอะซิเตต จากนั้นนำสารสกัดดังกล่าวไปศึกษาฤทธิ์ทางชีวภาพจากการศึกษาพบว่าสารสกัดทั้งหมดมีฤทธิ์ยับยั้งการสร้างไบโอฟิล์มของเชื้อ *Escherichia coli* ได้ดีเยี่ยม โดยขึ้นอยู่กับความเข้มข้นของสารสกัด การสร้างไบโอฟิล์มของเชื้อ *E. coli* ถูกยับยั้งมากกว่า 80% เมื่อบ่มด้วยสารสกัดชั้นไคลอโรฟอร์มและเอทิลอะซิเตตที่ความเข้มข้น 1,024 ไมโครกรัม/มิลลิเมตร โดยเปรียบเทียบกับชุดควบคุม ส่วนสกัดชั้นเฮกเซนพบว่าสามารถยับยั้งการสร้างไบโอฟิล์มของเชื้อดังกล่าวได้ โดยเชื้อสร้างไบโอฟิล์มได้น้อยกว่า 40% เมื่อบ่มด้วยสารสกัดที่ความเข้มข้น 1,024 ไมโครกรัม/มิลลิเมตร นอกจากนี้สารสกัดทั้งสามชนิดสามารถทำลายไบโอฟิล์มที่สร้างแล้วโดยทดสอบที่ความเข้มข้น 1,024 ไมโครกรัม/มิลลิเมตร ส่วนการศึกษาฤทธิ์ต้านอนุมูลอิสระพบว่าสารสกัดชั้นเอทิลอะซิเตตมีฤทธิ์ที่ดีมากในการต้านอนุมูล DPPH โดยมีค่า IC<sub>50</sub> เท่ากับ 22.7 ไมโครกรัม/มิลลิเมตร ซึ่งเป็นค่าที่มีความใกล้เคียงกับค่า IC<sub>50</sub> ของ BHT (24.3 ไมโครกรัม/มิลลิเมตร) นอกจากนี้สารสกัดชั้นไคลอโรฟอร์มและเอทิลอะซิเตตมีฤทธิ์ที่ดีในการต้านการอักเสบ โดยมีค่า IC<sub>50</sub> เท่ากับ 65.7 และ 66.9 ไมโครกรัม/มิลลิเมตรตามลำดับ จากผลการทดลองทั้งหมดแสดงให้เห็นว่าต้นพริกไทยมีฤทธิ์ทางชีวภาพที่หลากหลาย จึงสามารถเป็นแหล่งใหม่ของสารออกฤทธิ์ต้านไบโอฟิล์ม ฤทธิ์ต้านการอักเสบและฤทธิ์ต้านอนุมูลอิสระได้

**คำสำคัญ:** ต้นพริกไทย, ฤทธิ์ต้านไบโอฟิล์ม, ฤทธิ์ต้านการอักเสบ, ฤทธิ์ต้านอนุมูลอิสระ

### **1. Introduction**

In recent years, the use of medicinal plants in the prevention and treatment of diseases has gained considerable importance (Selvamangai & Bhaskar, 2012). *Eupatorium ayapana* (Syn. *Ayapana triplinerve* Vahl. and *Eupatorium*

*triplinerve* Vahl.) belongs to Asteraceae family and is one of the most important plants used in herbal medicine. It is an ornamental erect perennial herb with aromatic leaves and reddish brown stems. The medicinal plant is native to South America and can be found in other tropical countries such as

Hawaii, India, and Vietnam (Gauvin-Bialecki & Marodon, 2009).

According to its ethnopharmacological use, the plant is widely used as folk medicine in India for the immediate arrest of bleeding from wounds (Rajasekaran, Kalaivani, & Ariharasivakumar, 2010). In Indonesia, the leaves of *E. ayapana* are tropically used for skin care in combination with other herbs (Arung, Kuspradini, Kusuma, Shimizu, & Kondo, 2012). Moreover, the plant extracts have been reported to exhibit anti-venom (Maiti & Mishra, 2000), anti-inflammatory (Parimala, Cheriyan, & Viswanathan, 2012), antimicrobial (Narayanan et al., 2011; Unnikrishnan et al., 2014; Sugumar, Karthikeyan, & Gowdhami, 2015), anti-oxidant (Bepari, Maity, Sinha, & Choudhury, 2013; Melo et al., 2013; Krishnan, Jayaraj, Megala, & Elangovan, 2014; Sharath, Harish, Channarayappa, Preetham, & Sushma, 2014), hepatoprotective (Bose et al., 2007), anti-nociceptive (Melo et al., 2013; Parimala et al., 2012), and haemostatic (Rajasekaran et al., 2010) properties. However, anti-biofilm formation of the medicinal plant has not been evaluated.

Biofilms are communities of microorganisms attached to a surface. All biofilms, regardless of their location, share several common features. These include the synthesis of an extracellular polymeric matrix that holds the bacterial cells together (Kaplan, 2010). Biofilms are more resistant to antimicrobial agents compared to free-living or planktonic cells (Mah & O'Toole, 2001). In addition, implanted medical devices including intravenous catheters, artificial joints and, cardiac pacemakers are prime targets for bacterial biofilm formation (Donlan & Costerton, 2002; Parsek & Singh, 2003). The inherent protective nature of the biofilm colony makes most biofilm-associated infections difficult or impossible to eradicate (Kaplan, 2010). Consequently, effective agents are necessary to control biofilm-producing bacteria.

Therefore, the present study has been designed to investigate *E. ayapana* leaf extracts for their anti-biofilm formation and biological activities.

## 2. Materials and methods

### 2.1 Preparation of *E. ayapana* extracts

The air-dried ground leaves of *E. ayapana* (50 g) were extracted with ethanol (3 x 400 ml) in

a Soxhlet extractor. The ethanolic extract (10.56 g) was successively partitioned with hexane, CH<sub>2</sub>Cl<sub>2</sub>, and EtOAc and evaporated to obtain hexane, CH<sub>2</sub>Cl<sub>2</sub> and EtOAc extracts with yields of 2.12, 0.98, and 0.52 g, respectively. All of the extracts were kept at 4°C for future study on antimicrobial, anti-inflammatory, and anti-oxidant activities.

### 2.2 Bacterial strains and growth conditions

Bacterial strains including *Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 35984, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 10145 were used in this study. All bacterial strains were cultured on Mueller-Hinton agar (MHA) and incubated at 37°C for overnight. The pathogens were suspended in Mueller-Hinton broth (MHB) and incubated at 37°C for 3-5 h, and turbidity was adjusted to McFarland standard number 0.5 with 0.85% NaCl solution to achieve a concentration of approximately  $1.5 \times 10^8$  colony forming units (CFU)/ml.

### 2.3 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A modified broth microdilution method outlined by Clinical and Laboratory Standards Institute (CLSI) was performed (CLSI, 2011). The plant extracts were dissolved in 10% dimethyl sulphoxide (DMSO) and diluted two-fold to give final concentrations ranging from 0.5-1,024 µg/ml. One hundred microliters of the bacterial suspensions, containing approximately  $10^6$  CFU/ml of the microorganism, was inoculated in 80 µl of MHB supplemented with 20 µl of the extracts. The microtiter plates were incubated at 37°C for 16-18 h. Minimum inhibitory concentration was recorded as the lowest concentration of the extracts that was not permitted for any turbidity of the tested organism. Aliquots from the broth with no growth were spread onto fresh MHA plates using a sterile loop and incubated at 37°C overnight. The MBC was the lowest concentration that produced a complete kill of the microorganism. Vancomycin and gentamicin were included as positive controls.

### 2.4 Effect of *E. ayapana* extracts on biofilm formation

An experiment was performed according to the protocol of Karaolis et al. (2005) with slight modifications. Briefly, *E. ayapana* extracts were dissolved in 10% DMSO and diluted two-fold dilution in 96-well plates to give final concentrations that ranged from 64-1,024 µg/ml. One hundred microliters of *E. coli* suspension, containing approximately 10<sup>6</sup> CFU/ml of the microorganism in tryptic soy broth (TSB) supplemented with 0.25% glucose, was transferred to the 96-well plate containing 20 µl of *E. ayapana* extracts and 80 µl of TSB supplemented with 0.25% glucose. The microtiter plates were incubated at 37°C for 24 h. After incubation, the wells were washed twice with PBS to remove free-living cells, air-dried and stained with 200 µl of 0.1% crystal violet solution for 30 min. The plates were washed twice with distilled water, air-dried, and dissolved with 200 µl of DMSO. Biofilm formation was measured at OD 570 nm using a microplate reader. One per cent (1%) DMSO was included as a negative control. The relative percentage of biofilm formation was defined as: (mean OD 570 nm of treated well/mean OD 570 nm of control well) x100.

#### 2.5 Effect of *E. ayapana* extracts on established biofilms

Established biofilms of *E. coli* were grown as described by Kuzma, Rozalski, Walencka, Rozalska, and Wysokinska, 2007. Two hundred microliters of an *E. coli* suspension, containing approximately 10<sup>6</sup> CFU/ml of the microorganism in TSB supplemented with 0.25% glucose, was transferred to the 96-well plate. The microtiter plates were incubated at 37°C for 24 h. After incubation, the medium was removed and the

wells were rinsed twice with PBS. TSB supplemented with 0.25% glucose (180 µl) and 20 µl of *E. ayapana* extracts at different concentrations (64-1,024 µg/ml) were added. After incubation, the established biofilm was stained and measured as described above.

#### 2.6 Inhibitory effects on LPS-induced nitric oxide (NO) production from RAW264.7 cells

RAW264.7 cells were seeded into 96-well plates at 1 x 10<sup>5</sup> cells/well and allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The medium was replaced with a fresh medium containing 100 ng/ml of LPS (from *E. coli*, 055:B5) together with the test samples at various concentrations (3-100 µg/ml) and incubated for 24 h. NO synthase inhibitor (L-NA), NF-κB inhibitor (CAPE) and non-steroidal anti-inflammatory drugs, NSAIDs (indomethacin) were used as positive controls. The stock solution of each test sample was dissolved in 1% DMSO, and the solution was added to the medium RPMI.

NO production by RAW264.7 cells was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent as previously described (Tewtrakul, Subhadhirasakul, Karalai, Ponglimanont, & Cheenpracha, 2009). After 24 h of incubation, cells generated NO in the medium, and the supernatants (100 µl) were collected and reacted with Griess reagent (100 µl). NO production was measured spectrophotometrically at 570 nm using a microplate reader. The percent inhibition was calculated based on the following equation and IC<sub>50</sub> values were determined graphically (*n* = 4):

$$\text{Inhibition (\%)} = [(A - B) / (A - C)] \times 100$$

A-C: NO<sub>2</sub><sup>-</sup> concentration (µM)

[A: LPS (+), sample (-); B: LPS (+), sample (+); C: LPS (-), sample (-)]

#### 2.7 Viability assay of RAW264.7 macrophage cells

Viability of RAW264.7 cells was assayed using the MTT colorimetric method after 24 h incubation with various concentrations of test samples. This method requires active mitochondria of living cells to reduce MTT, a pale yellow substrate to yield a dark blue formazan product. Briefly, MTT solution (10 µl, 5 mg/ml) was added to the wells and further incubated for 4 h at 37°C in a humidified atmosphere containing

5% CO<sub>2</sub>. The media was removed, the formazan products made due to dye reduction by viable cells were dissolved using DMSO and the optical density was measured with a microplate reader at a wavelength of 570 nm. The test samples were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control group.

#### 2.8 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) scavenging assay

The methodology described by Jitsanong et al. (2011) to assess the DPPH free radical scavenging capacity with slight modifications (Jitsanong, Khanobdee, Piyachaturawat, Wongprasert, 2011). The stock solution (10 µg/ml) of the sample was prepared in DMSO and diluted to concentrations ranging from 1-200 µg/ml with absolute ethanol. The reaction mixture contained 100 µl of samples at various concentrations and 100 µl of 0.1 mM DPPH in absolute ethanol. Butylated hydroxytoluene

(BHT) and quercetin were used as positive controls. The DPPH solution in the absence of sample was used as a control and absolute ethanol was used as a blank. The bleaching was measured at 517 nm using a microplate reader after incubation for 30 min in the dark condition. The percentage of scavenging activity of the sample against DPPH radical was calculated according to the following equation and IC<sub>50</sub> values were determined graphically (*n* = 4):

$$\% \text{ Inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

A<sub>control</sub> = Absorbance of control - Absorbance of control blank  
 A<sub>sample</sub> = Absorbance of sample - Absorbance of sample blank

### 3. Results and discussion

#### 3.1 Antibacterial activity and anti-biofilm formation

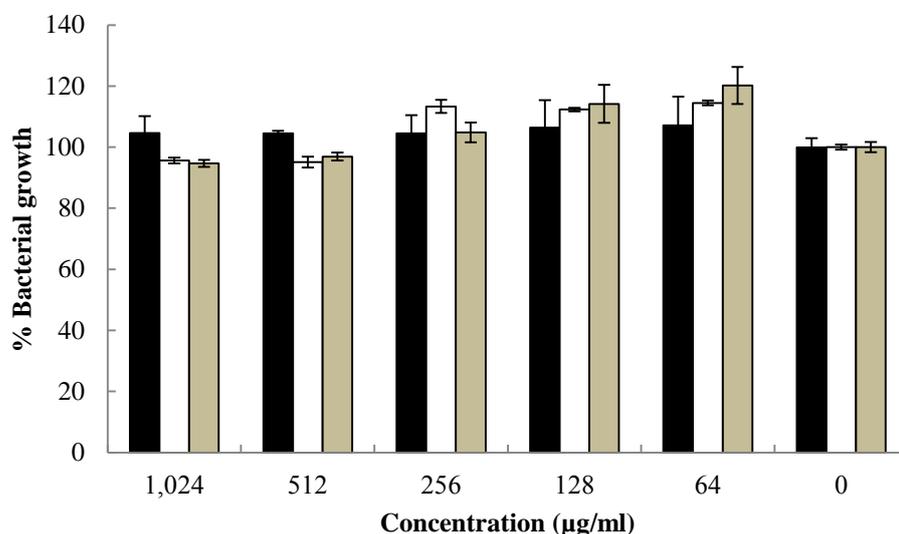
*E. ayapana* leaves were collected from Ayuthaya province, Thailand in December 2013 and extracted with ethanol using Soxhlet extractor. The ethanolic extract was further partitioned with hexane, CH<sub>2</sub>Cl<sub>2</sub>, and EtOAc solvents. The results demonstrated that the highest yield was observed in hexane fraction (2.12 g). Facknath and Lalljee (2008) reported that the highest % yield of the fresh young leaves was obtained from hexane fraction (3%). In contrast, the hexane fraction exhibited the lowest amounts of alkaloids, sterols, terpenes, phenols, tannins, and flavonoids compared with petroleum ether and CHCl<sub>3</sub>-methanol fractions. Moreover, Parimala et al. (2012) demonstrated that the presence of sterols, carbohydrates, tannins, phenols, glycosides, and alkaloids was detected in the petroleum ether extract.

*In vitro* antibacterial activity of *E. ayapana* extracts against human pathogens was evaluated using broth microdilution method. The activities of the extracts against human pathogens are indicated in Table 1. All of the extracts

exhibited weak antibacterial properties against the tested microorganisms. The MIC and MBC values of the extracts against Gram-positive and Gram-negative pathogens ranged from 512 to >1,024 µg/ml. The results suggested that all of the extracts showed no antibacterial activity against *E. coli* (MIC/MBC >1,024 µg/ml). The results correlated with the bacterial growth study as demonstrated in Figure 1. *E. ayapana* extracts elucidated no inhibition effect on bacterial growth after incubation with the extract for 24 h. In 2011, Narayanan et al., 2011, reported that *E. ayapana* methanolic extract showed no activity against multiple antibiotic resistant uropathogens. Begum and co-workers (2010) showed that essential oil from *E. ayapana* aerial parts extracts exhibited weak antibacterial and antifungal activities against 10 bacterial strains and six phytopathogenic fungi with MIC values ranging from 6,000-21,000 ppm. Rahman and Junaid (2008) reported that CHCl<sub>3</sub> extract of the leaves showed the largest zone of inhibition (22 mm in diameter with 1,000 µg/disc extract) against *Vibrio* spp. Moreover, the extract demonstrated moderate antibacterial activity against the pathogen with MIC value of 125 µg/ml.

**Table 1** Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Eupatorium ayapana* extracts against Gram-positive and Gram-negative pathogenic bacteria

Fraction	MIC/MBC (µg/ml)			
	<i>S. aureus</i> ATCC 25923	<i>S. epidermidis</i> ATCC 35984	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 10145
Hexane	>1,024/>1,024	>1,024/>1,024	>1,024/>1,024	>1,024/>1,024
CH <sub>2</sub> Cl <sub>2</sub>	512/>1,024	1,024/>1,024	>1,024/>1,024	1,024/>1,024
EtOAc	1,024/>1,024	1,024/>1,024	>1,024/>1,024	1,024/>1,024
Vancomycin	0.5/0.5	0.5/1	-	-
Gentamicin	-	-	0.5/1	1/4

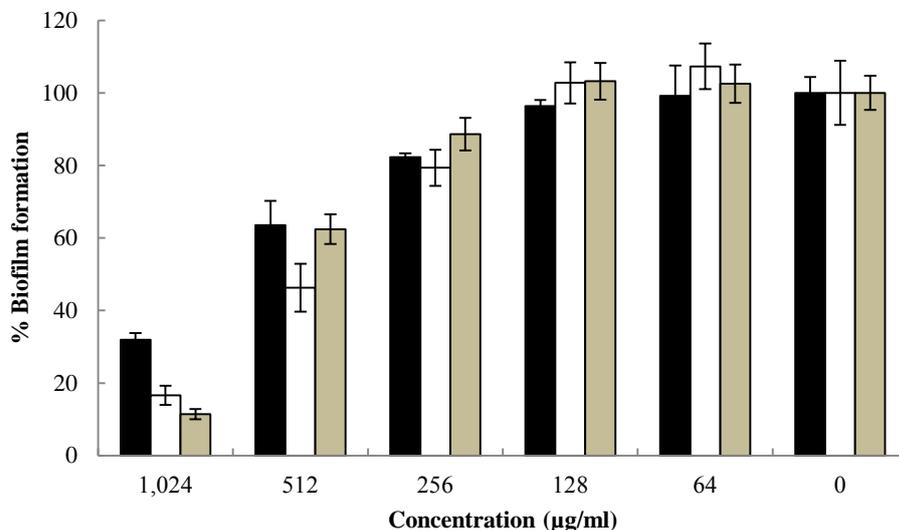


**Figure 1** Bacterial growth of *Escherichia coli* ATCC 25922 after treatment with various concentrations of hexane (black bars), CH<sub>2</sub>Cl<sub>2</sub> (open bars) and EtOAc (shaded bars) extracts of *Eupatorium ayapana*. 1% DMSO was included as negative control. The results are shown as means  $\pm$  S.E.M. of triplicate results.

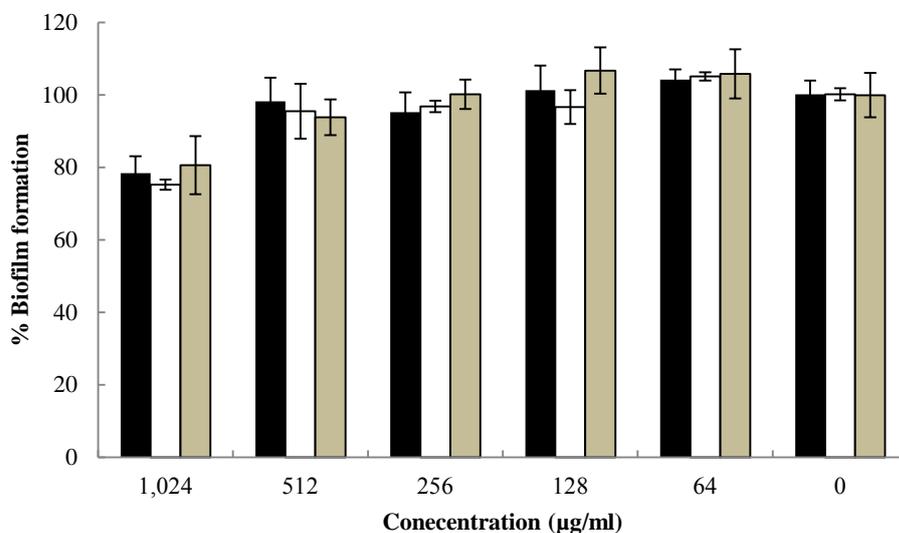
In the present study it was found that *E. ayapana* extracts exhibited excellent anti-biofilm formation of *E. coli* (Figure 2). *E. coli* biofilm formation was inhibited more than 80% after treatment with the CH<sub>2</sub>Cl<sub>2</sub>, and EtOAc extracts (1,024 µg/ml) compare with untreated cells. In addition, the microorganism produced biofilm 40% less after treatment with 1,024 µg/ml hexane extract. Treatment of all of the extracts at 512 and 256 µg/ml was found to be effective in reducing *E. coli* biofilm formation by approximately 40% and 20%, respectively. However, all of the extracts at concentration 64-256 µg/ml showed only a slight effect on biofilm formation. The impact of the extracts on biofilm formation was found to be dose-dependent.

For anti-established biofilm, the organism at mid-exponential phase was culture to produced biofilm for 24 h and then exposed to various concentrations of *E. ayapana* extracts. The results indicated that the addition of *E. ayapana* extracts (1,024 µg/ml) to the culture also resulted in decreased established bacterial biofilm. However,

all of the leaves extracts at concentration 64-512 µg/ml had little effect on the established biofilm of the bacterial strain (Figure 3). More than 70 years after the first report on biofilms (Zobell, 1943), they are still a concern in a broad range of areas especially biomedical fields. Biofilm development can be divided into three distinct stages: attachment of cells to a surface, formation of a multilayered cell cluster surrounded by an extracellular polysaccharide matrix, and detachment of cells from the colony into the surrounding medium (Kaplan, 2010). The production of an extracellular polysaccharide matrix constitutes a protected mode of growth that allows microorganisms to survive in hostile environments and it has become clear that biofilm-grown cells express properties distinct from planktonic cells (Mah & O'Toole, 2001). Therefore, the emergence of resistant bacteria to conventional antimicrobial agents clearly demonstrates that new biofilm control strategies are required (Simoes, Simoes & Vieira, 2010).



**Figure 2** Anti-biofilm formation of *Escherichia coli* ATCC 25922 after treatment with various concentrations of hexane (black bars), CH<sub>2</sub>Cl<sub>2</sub> (open bars) and EtOAc (shaded bars) extracts of *Eupatorium ayapana*. 1% DMSO was included as negative control. The results are shown as means ± S.E.M. of triplicate results.



**Figure 3.** Anti-established biofilm formation of *Escherichia coli* ATCC 25922 after treatment with various concentrations of hexane (black bars), CH<sub>2</sub>Cl<sub>2</sub> (open bars) and EtOAc (shaded bars) extracts of *Eupatorium ayapana*. 1% DMSO was included as negative control. The results are shown as means ± S.E.M. of triplicate results.

### 3.2 *In vitro* toxicity and anti-inflammation

Cytotoxicity testing was performed in RAW264.7 cells and the results demonstrated that all of the extracts were non-toxic to the tested cells at a concentration of 100 µg/ml. Moreover, the CH<sub>2</sub>Cl<sub>2</sub> and EtOAc extracts exhibited moderate anti-inflammatory activity against nitric oxide in

RAW264.7 cells with IC<sub>50</sub> values of 65.7 and 66.9 µg/ml, respectively (Table 2). In 2013, Melo et al. evaluated acute oral toxicity of *E. ayapana* ethanolic extracts in mice. The results showed that no death was observed after treatment with 2,000 mg/kg and 5,000 mg/kg of the extract dosage for 14 days. This result was similar to Parimala et al.

(2012), who reported that the petroleum ether extract was non-toxic up to a maximum dose of 2,000 mg/kg body weight of mice. Moreover, the extract exhibited anti-nociceptive and anti-inflammatory activities *in vivo*. The extract exhibited significant inhibition of acetic acid

induced writhing and carrageenan induced hind paw edema in rats (Parimala et al., 2012). In addition, the ethanolic extract has been reported as a mild sedative, anxiolytic, and showed anti-depressive effects on the central nervous system (Melo et al., 2013).

**Table 2** Anti-inflammatory activity of *Eupatorium ayapana* extracts on inhibition of nitric oxide production in RAW264.7 cells

Fraction	% inhibition at various concentration (µg/ml)						IC <sub>50</sub> (µg/ml)
	0	1	3	10	30	100	
Hexane	0.0±1.7	-	-	17.1±1.3	22.6±0.7	44.2±0.5	>100
CH <sub>2</sub> Cl <sub>2</sub>	0.0±1.7	-	-	16.2±0.9	26.3±1.0	62.3±0.4	65.7
EtOAc	0.0±1.7	-	-	17.1±1.5	26.7±1.6	61.5±2.3	66.9
L-NA	0.0±2.4	-	-	20.0±2.3	35.5±1.7	90.0±0.8	32.6
CAPE	0.0±2.4	35.9±1.7	62.0±1.7	74.0±1.0	84.9±0.8	104.5±0.8*	2.0
Indomethacin	0.0±2.4	-	4.8±1.5	16.7±2.0	38.5±3.0	87.2±0.3	33.8

\*Cytotoxic effect was observed. Value represents mean ± S.E.M. (n=4).

### 3.3 Anti-oxidant activity

For anti-oxidant activity, the EtOAc extract of *E. ayapana* investigated showed pronounced activity against DPPH radical with IC<sub>50</sub> value at 22.7 µg/ml, which is very close to that of BHT (24.3 µg/ml). In addition, IC<sub>50</sub> values of the CH<sub>2</sub>Cl<sub>2</sub> and hexane against DPPH radicals were 83.9 and >200 µg/ml, respectively (Table 3). The results suggested that anti-oxidant agents may

be obtained from EtOAc fraction. Bepari and co-workers (2013) showed that *E. ayapana* leaf extracts exhibited enhanced anti-oxidant potential in Ehrlich's ascites carcinoma-bearing Swiss albino mice. Moreover, the aerial part extract decreased Trolox equivalent anti-oxidant capacity, nitric oxide, and malondialdehyde levels in response to swimming stress induced in rats (Melo et al., 2013).

**Table 3** Antioxidant activity of *Eupatorium ayapana* extracts against DPPH radical

Fraction	% inhibition at various concentration (µg/ml)									IC <sub>50</sub> (µg/ml)
	0.78	1.56	3.13	6.25	12.5	25	50	100	200	
Hexane	-	-	-	-	22.8±1.3	36.3±2.1	61.1±2.4	-7.6±3.2	-0.2±3.1	>200
CH <sub>2</sub> Cl <sub>2</sub>	-	-	-	-	1.6±0.8	12.8±1.3	29.7±1.7	49.9±0.7	82.0±0.8	83.9
EtOAc	-	-	-	-	30.4±1.8	53.4±1.7	76.9±1.4	88.8±0.4	100.7±1.0	22.7
BHT	1.5±0.3	5.9±0.1	11.0±0.9	21.8±0.1	35.9±0.7	52.7±0.1	68.8±1.4	79.5±0.4	88.4±0.5	24.3
Quercetin	19.7±1.3	42.2±2.6	73.6±4.7	90.1±1.0	91.8±0.2	92.7±0.1	91.6±1.3	93.0±0.1	94.1±0.4	1.8

Value represents mean ± S.E.M. (n=4).

## 4. Conclusions

The hexane, CH<sub>2</sub>Cl<sub>2</sub>, and EtOAc extracts exhibited pronounced anti-biofilm formation against *E. coli* in a dose-dependent manner. Moreover, the EtOAc fraction demonstrated excellent anti-oxidant activity against DPPH radicals. In addition, the CH<sub>2</sub>Cl<sub>2</sub> and EtOAc extracts showed good anti-inflammatory activity without toxicity to RAW264.7 cells. The apparent difference in the biological activities of the extracts can be used as novel anti-biofilm, anti-inflammatory, and anti-oxidant agents.

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## 6. References

- Arung, E. T., Kuspradini, H., Kusuma, I. W., Shimizu, K., & Kondo, R. (2012). Validation of *Eupatorium triplinerve* Vahl leaves, a skin care herb from East Kalimantan, using a melanin biosynthesis assay. *Journal of Acupuncture & Meridian Studies*, 5(2), 87-92. DOI: 10.1016/j.jams.2012.01.003
- Begum, J., Bhuiyan, N. I., & Taznin, T. (2010). Chemical composition and antimicrobial activity of essential oil from *Eupatorium triplinerve* Vahl. aerial parts. *Asian Journal of Microbiology, Biotechnology & Environmental Sciences*, 12, 543-547.
- Bepari, M., Maity, P., Sinha, B., & Choudhury, S. M. (2013). *Eupatorium ayapana* leaf extracts enhance anti-oxidant potential in Ehrlich's ascites carcinoma-bearing Swiss albino mice. *International Journal of Life science & Pharma Research*, 3(4), 1-10.
- Bose, P., Gupta, M., Mazumder, U. K., Kumar, R. S., Thangavel, S., & Kumar, R. S. (2007). Hepatoprotective and anti-oxidant effects of *Eupatorium ayapana* against carbon tetrachloride induced hepatotoxicity in rats. *Iranian Journal of Pharmacology & Therapeutics*, 6(1), 27-33.
- Clinical and Laboratory Standards Institute (CLSI). (2011). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard-eighth Edition*. Pennsylvania, USA: Clinical and Laboratory Standards Institute.
- Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, 15(2), 167-193. DOI: 10.1128/CMR.15.2.167-193.2002
- Facknath, S., & Lalljee, B. (2008). Study of various extracts of *Ayapana triplinervis* for their potential in controlling three insect pests of horticulture crops. *Tropicultura*, 26(2), 119-124.
- Gauvin-Bialecki, A., & Marodon, C. (2009). Essential oil of *Ayapana triplinervis* from Reunion Island: a good natural source of thymohydroquinone dimethyl ether. *Biochemical Systematics and Ecology*, 36(11), 853-858. DOI: 10.1016/j.bse.2008.09.006
- Jitsanong, T., Khanobdee, D., Piyachaturawat, P., & Wongprasert, K. (2011). Diarylheptanoid 7-(3,4 dihydroxyphenyl)-5-hydroxy-1-phenyl-(1E)-1-heptene from *Curcuma comosa* Roxb. protects retinal pigment epithelial cells against oxidative stress-induced cell death. *Toxicology in Vitro*, 25(1), 167-176. DOI: 10.1016/j.tiv.2010.10.014
- Kaplan, J. B. (2010). Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *Journal of dental research*, 89(3), 205-218. DOI: 10.1177/0022034509359403
- Karaolis, D. K., Rashid, M. H., Chythanya, R., Luo, W., Hyodo, M., & Hayakawa, Y. (2005). c-di-GMP (39-59-cyclic diguanylic acid) inhibits *Staphylococcus aureus* cell-cell interactions and biofilm formation. *Antimicrobial Agents and Chemotherapy*, 49(3), 1029-1038. DOI: 10.1128/AAC.49.3.1029-1038.2005
- Krishnan, M., Jayaraj, R. L., Megala, J., & Elangovan, N. (2014). Antioxidant mediated antiulcer effect of *Eupatorium triplinerve* Vahl against acetic acid induced ulcerative colitis in mice. *Biomedicine and Aging Pathology*, 4(2), 153-160. DOI: 10.1016/j.biomag.2013.12.002
- Kuzma, L., Rozalski, M., Walencka, E., Rozalska, B., & Wysokinska, H. (2007). Antimicrobial activity of diterpenoids from hairy roots of *Salvia sclarea* L.: salvipisone as a potential anti-biofilm agent active against antibiotic resistant staphylococci. *Phytomedicine*, 14(1), 31-35. DOI: 10.1016/j.phymed.2005.10.008
- Mah, T. F. C., & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microb*, 9(1), 34-39. DOI: 10.1016/S0966-842X(00)01913-2
- Maiti, S., & Mishra, T. K. (2000). Anti-venom drugs of Santals, Savars and Mahatos of Midnapore distric of West Bengal, India. *Ethnobotany*, 12, 77-80.
- Melo, A. S., Monteiro, M. C., Silva, j. B., Oliverra, F. R., Vieira, J. S. F., Andrade, M. A., . . . Maia, C, S. F. (2013). Antinociceptive,

- neurobehavioral and anti-oxidant effects of *Eupatorium triplinerve* Vahl on rats. *Journal of Ethnopharmacology*, 147(2), 293-301. DOI: 10.1016/j.jep.2013.03.002
- Narayanan, A. S., Raja, S. S. S., Ponmurugan, K., Kandekar, S. C., Natarajaseenivasan, K., Maripandi, A., & Mandeel, Q. A. (2011). Antibacterial activity of selected medicinal plants against multiple antibiotic resistant uropathogens: a study from Kolli Hills, Tamil Nadu, India. *Beneficial Microbes*, 2(3), 235-243. DOI: 10.3920/BM2010.0033
- Parimala K., Cherian, B. V., & Viswanathan, S. (2012). Antinociceptive and anti-inflammatory activity of petroleum ether extract of *Eupatorium triplinerve* Vahl. *International Journal of Life science & Pharma Research*, 2(3), 12-16.
- Parsek, M. R., & Singh, P. K. (2003). Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol*, 57, 677-701. DOI: 10.1146/annurev.micro.57.030502.090720
- Rahman, S., & Junaid, M. (2008). Antimicrobial activity of leaf extracts of *Eupatorium triplinerve* Vahl. against some human pathogenic bacteria and phytopathogenic fungi. *Bangladesh Journal of Botany*, 37(1), 89-92. DOI: 10.3329/bjb.v37i1.1570
- Rajasekaran, A., Kalaivani, M., & Ariharasivakumar, G. (2010). Haemostatic effect of fresh juice and methanolic extract of *Eupatorium ayapana* leaves in rat model. *International Journal of Biological & Medical Research*, 1(3), 85-87.
- Selvamangai, G., & Bhaskar, A. (2012). GC-MS analysis of phytochemicals in the methanolic extract of *Eupatorium triplinerve*. *Asian Pacific Journal of Tropical Biomedicine*, 2012, 1329-1332.
- Sharath, R., Harish, B. G., Channarayappa, S., Preetham, J., & Sushma, S. (2014). Evaluation of antioxidant activity of coumarin isolated from *Eupatorium triplinerve*. *Research and Reviews: a Journal of Pharmacognosy*, 1(1), 18-22.
- Simoës, M., Simoës, L. C., & Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT-Food Science Technology*, 43(4), 573-583. DOI: 10.1016/j.lwt.2009.12.008
- Sugumar, N., Karthikeyan, S., & Gowdhami, T. (2015). Chemical composition and antimicrobial activity of essential oil from *Eupatorium triplinerve* Vahl aerial parts. *International Letters of Natural Sciences*, 4, 14-21.
- Tewtrakul, T., Subhadhirasakul, S., Karalai, C., Ponglimanont, C., & Cheenpracha, S. (2009). Anti-inflammatory effects of compounds from *Kaempferia parviflora* and *Boesenbergia pandurata*. *Food Chemistry*, 115(2), 534-538. DOI: 10.1016/j.foodchem.2008.12.057
- Unnikrishnan, P. K., Varughese, T., Sreedhar, S., Balan, N., Balachandran, I., & Shree, A. B. (2014). Study on *Eupatorium triplinerve* Vahl from South India, a rich source for thymohydroquinone dimethylether and its antimicrobial activity. *Journal of Essential Oil-Bearing Plants*, 17, 652-657. DOI: 10.1080/0972060X.2014.914000
- Zobell, C. E. (1943). The effect of solid surfaces upon bacterial activity. *Journal of Bacteriology*, 46(1), 39-56.