EVALUATION OF ANTIBACTERIAL EFFECT OF *PHALERIA MACROCARPA* EXTRACT AGAINST BACTERIAL SPECIES ISOLATED FROM HUMAN DIABETIC WOUND INJURIES USING SCANNING ELECTRON MICROSCOPY

Hanan Kumar Gopalan[1], Noman D Salih*[1], Farah Hanim Roslan[1], Norfatin Azmi[1], Hing, H. L[2]

[1]*Universiti Kuala Lumpur, Institute of Medical Science Technology, Blok A1-1, Jalan TKS 1, Taman Kajang Sentral, 43000 Kajang, Selangor Darul Ehsan, Malaysia*

[2] *School of Diagnostic & Applied Health Sciences, Faculty of Health Science, Universiti Kebangsaan Malaysia, 50300 Jalan Raja Muda Kuala Lumpur, Malaysia*

ABSTRACT: *This study has been designed to investigate the protective effects of Phaleria macrocarpa methanol extract, and to evaluate its antimicrobial activity against common pathogenic microbes isolated from human diabetic wounds using scanning electron microscopy (SEM). Escherichia coli, Klebsiella pneumoniaea, Pseudomonas mallei, Klebsiella aerogenes and Proteus sp were isolated/identified and tested against the plant extract at different concentrations using the MIC and MBC techniques and observed using scaning electrone microscope(SEM). Results obtained showed that the use of the highest concentration (100%) of extract totally inhibits the growth of P. mallei, E. coli, K. pneumoniae, and K. aerogenes, but resisitance were observed in Proteus sp .Observation under SEM reveals that the antimicrobial activity of the extract against tested bacteria demonstrated cell damage, elongation of cells and inhibitions of cell division. These findings have revealed the effectiveness of the extract against these microbes, as well as, the antimicrobial potency to inhibit and damage bacterial cells, which we believe that it could be used to develop a promising natural antimicrobial agent, that serves to avoid, treat, or lessen most of the complications of wound infections especially seen in diabetic patients.*

Keywords:*Phaleriamacrocarpa*, Antimicrobial Effects, Scanning Electron Microscope, Diabetic Wound.

Corresponding: Dr. Noman D Salih, Clinical Laboratory Science Sec., Institute of Medical Science & Technology (Mestech), Universiti Kuala Lumpur (UniKL). Email: nomand@unikl.edu.my ; Tel: +6012 319 0051

1. INTRODUCTION

Diabetes mellitus (DM) is a chronic disorder caused by inheritance or acquired deficiency, which develops either due to insufficient production of insulin by the pancreas, or the ineffectiveness of the peripheral insulin hormone produced. It affects nearly 4% of the population worldwide, but this ratio is expected to increase by 5.4% in 2025 [1] and up to 7.7% (439 million adults) by 2030 [2]. DM is a multisystemic chronic disease frequently complicated by complex wound infections, due to the slowing down ability of the body to fight infections because of hyperglycemia that can lead to higher levels of glucose in the peripheral tissues, allowing bacteria to grow and infection to develop rapidly [3]. Various medicinal plants have been used for years in daily life to treat several diseases, and it has been estimated that about four billion people (80% of world population) presently use herbal medicine for primary healthcare, mostly in India and China which still relies on traditional medicines [4]. *Phaleria macrocarpa*, is a plant from the family of Thymelaeaceae, commonly known as "God's Crown" or Mahkota Dewa, which was believed traditionally that its fruits were descended from the sky because of prayers offered by the Divine servants of God [5]. Its seeds were brought by ancient traditional healers from the Island of Papua and were planted in the open areas of Solo and Yogyakarta in Indonesia, as well as, the northwestern part of Malaysia [6]. In traditional medicine ,the seeds is believed to contribute to the well being of human health. Additionly,the extracts have been reported for numeourous valuable medicinal properties, such as anti-cancer, anti-diabetic, anti-inflammatory, anti-fungal, anti-oxidant, anti-bacterial, and vasorelaxant activities [7]. Our phytochemical screening of the metahanol leaves extract showed the presence of alkaloid, flavonoid, saponin, tannins, reducing sugar, terpenoids, cardiac glycosides and phenolic compound . Therefore, this study was designed to investigate the protective effects of this extract and to evaluate its antimicrobial activity against pathogenic bacteria isolated from human diabetic wounds using scanning electron microscope (SEM).

2. MATERIALS AND METHOD

2.1 Patients samples

Serial swabs of 20 diabetic wound samples were obtained from each wound of the diabetic patients who underwent treatment at the local hospital in Kuala Lumpur Malaysia.

2.2 Bacteria Isolation and Identification

Sampels were cultivated and bacterial species were isolated and identified using biochemical test and Gram's staining. Five common bacteria obtained were *Pseudomonas mallei*, *Klebsiella pneumoniae*, *Escherichia coli*, *Klebsiella aerogenes* and *Proteus sp*. Stock cultures were maintained on nutrient agar slant and then sub-cultured in peptone water at 37°C prior to each antimicrobial testing .

2.3 Plant Extract

Fresh leaves of *P. macrocarpa* were collected from the northwestern part of Malaysia (Kedah), and taxonomically identified. The selected plant parts were dried, crushed in an electric grinder and pulverized into a coarse powder. Methanolic extraction was prepared by soaking 100g of the coarse powder in a conical flask with 500 ml of absolute methanol. The mixture was kept for 36 hours and stirred intermittently at 4 hours interval. It was then filtered, and dried under low pressure using rotary evaporator fitted with vacuum pump [8-11]. At the end of the drying process the paste obtained were dissolved in normal saline at different concentrations of 100%, 90%, 80%, 70%, 60%, and 50%.

2.4 Antibacterial assay

To calculate the minimal inhibitory concentration (MIC), serial tubes of *P. macrocarpa* extract was mixed with peptone water to achieve a final concentrations of 100%, 90%, 70%, 60%, and 50%.Then each species of bacteria were added into each test tube and incubated at 37°C at different time

intervals of 0,1,3,5,24,48 hours.Then bacterial cultures were inoculated into Mueller Hinton agar to determine the minimal bacterial concentration (MBC).

2.5 Scanning Electron Microscopy

Bacterial species susceptible to the plant extraction were used for scanning electron microscope (SEM) observation. Cover slip was applied to the surface area of the inhibition zone of agar plate and left for 5 minutes. In addition, a 1 sq. cm of agar was cut and fixed in 3% (v/v) glutaraldehyde buffer solution in 0.1 M sodium phosphate buffer (pH 7.2) for 24 hours. The samples were then washed with sodium phosphate buffer for three times, followed by dehydration in a serial ascending concentrations of alcohol, 30%, 50%, 70%, 80%, 90%, 95% and 100%. The specimens were then dried and mounted onto stubs and the samples, then observed in Hitachi SU3500 Scanning Electron Microscope.

3. RESULTS

3.1 Antibacterial assay of *P. macrocarpa* extract

Growth of *E.coli*, *Pseudomonas mallei*, *Klebsiella pneumoniae*, and *Klebsiella aaerogenes*, were inhibited at the higher concentrations of the extract (Table1-4) while *proteus sp.* (Table 5) showed resisitance at all concentration of extracts at different time intervals.

3.2 Scanning Electron microscopy (SEM)

Bacterial cells treated with extract were compared with untreated cells (control). Results showed that treated cells appeared shrunk and degradation of the cell wall was observed with noticeable damage to the outer layer of the cell wall. The extract was also able to inhibit the growth of the bacteria, as shown by a decrease in amount and elongation of bacterial cells (Figure 1).

Table 1: Antimicrobial effects of *P. macrocarpa* extract against *Pseudomonas mallei*

Pseudomonas mallei

Time	+ve	(-ve) Gentamycin	Extract concentration %					
			100%	90%	80%	70%	60%	50%
0 hours	+	+	+	+	+	+	+	+
1 hours	+	+	-	-	+	+	+	+
3 hours	+	-	-	-	-	+	+	+
5 hours	+	-	-	-	-	-	-	-
24 hours	+	-	-	-	-	-	-	-
48 hours	+	-	-	-	-	-	-	-

P. mallei showed obvious inhibition within 1hr at a concentration of 100% extract

Table 2: Antimicrobial effects of *P. macrocarpa* extract against *E. coli*

Escherichia coli

Time	(+ve)	(-ve) Gentamycin	Extract concentration %					
			100%	90%	80%	70%	60%	50%
0 hours	+	+	+	+	+	+	+	+
1 hours	+	-	-	+	+	+	+	+
3 hours	+	-	-	-	-	-	-	+
5 hours	+	-	-	-	-	-	-	-
24 hours	+	-	-	-	-	-	-	-
48 hours	+	-	-	-	-	-	-	-

E.coli showed obvious inhibition within 1hr at a concentration of 100% extract

Table 3: Antimicrobial effects of *P. macrocarpa* extract against *Klebsiella pneumoniae*

Klebsiella pneumoniae

Time	(+ve)	(-ve) Gentamycin	Extract concentration %					
			100%	90%	80%	70%	60%	50%
0 hours	+	+	+	+	+	+	+	+
1 hours	-	+	-	-	-	-	+	+
3 hours	-	-	-	-	-	-	-	+
5 hours	-	-	-	-	-	-	-	-
24 hours	-	-	-	-	-	-	-	-
48 hours	-	-	-	-	-	-	-	-

*Klebssiella pneumonia*e showed inhibition at all concentration after 5 hours up to 48 hours.

Table 4: Antimicrobial effects of *P. macrocarpa* extract against *Klebsiella aerogenes*

Klebsiela aerogenes

Time	+ve	(-ve) Gentamycin	Extract concentration %					
			100%	90%	80%	70%	60%	50%
0 hours	+	+	+	+	+	+	+	+
1 hours	+	+	+	+	+	+	+	+
3 hours	+	-	+	+	+	+	+	+
5 hours	+	-	-	-	+	+	+	+
24 hours	+	-	-	-	-	-	-	-
48 hours	+	-	-	-	-	-	-	-

Klebsiellan aerogenes showed strong inhibition at all concentration after 24 hours of incubation.

Table 5: Antimicrobial effects of *P. macrocarpa* extract against *Proteus sp.*

Proteus sp.

Time	(+ve)	(-ve) Gentamycin	Extract concentration %					
			100%	90%	80%	70%	60%	50%
0 hours	+	+	+	+	+	+	+	+
1 hours	+	+	+	+	+	+	+	+
3 hours	+	+	+	+	+	+	+	+
5 hours	+	+	+	+	+	+	+	+
24 hours	+	+	+	+	+	+	+	+
48 hours	+	-	+	+	+	+	+	+

Proteus sp. showed resistance at all concentration of extract.

4. DISCUSSION

This study evaluated the protective effects of *P. macrocarpa* leaves extract against bacteria species isolated from human diabetic wound injuries. However, different extractions of different parts of this plant have been reported previously for their valuable medicinal properties, such as anti-cancer, anti-diabetic, anti-inflammatory, anti-fungal, anti-oxidant, anti-bacterial, and vasorelaxant activities.The evaluation and the investigation of the antibacterial effectiveness of methanol leaves extract of *P.macrocarpa* on pathogenic bacterial cells using *SEM* technique was only done in this present study.

Diabetes mellitus is known to slow down the ability of the body to fight off infections due to the hyperglycemic state where high levels of glucose in the peripheral tissues favors and accelerate the growth bacteria and thus leading to infections that develops rapidly [12]. This study had revealed the antibacterial properties of *P. macrocarpa* leaves extraction against selected pathogenic bacteria using minimal bactericidal concentrations, which is in agreement with previous studies reported the antibacterial properties of different extractions of other parts of this plant [13-17]. In the current study, the antibacterial potencies of this extract have been supported, by SEM observation showing that the extract was effective to cause morphological alterations on the cell wall, and thus, revealed its antimicrobial mechanism by causing severe lysis and degradation of bacterial cell wall.

CONCLUSION

P. macrocarpa leaves methanolic extraction showed antimicrobial activities against five pathogenic bacterial species, by causing degradation of cell wall and shrinkage of the bacterial cells. Furthermore, these results suggest the possibility of testing *P. macrocarpa* leaves extraction against some broad spectrum microbes, and this effect might be increased by increasing the quantity and the concentration of the extract.Hence, this might be a promising compound to be used to develop a natural antimicrobial agent that could control antibiotic-resistant bacteria and serves to avoid, treat, or lessen most of the complications of wound injury infections especially seen in diabetic patients.

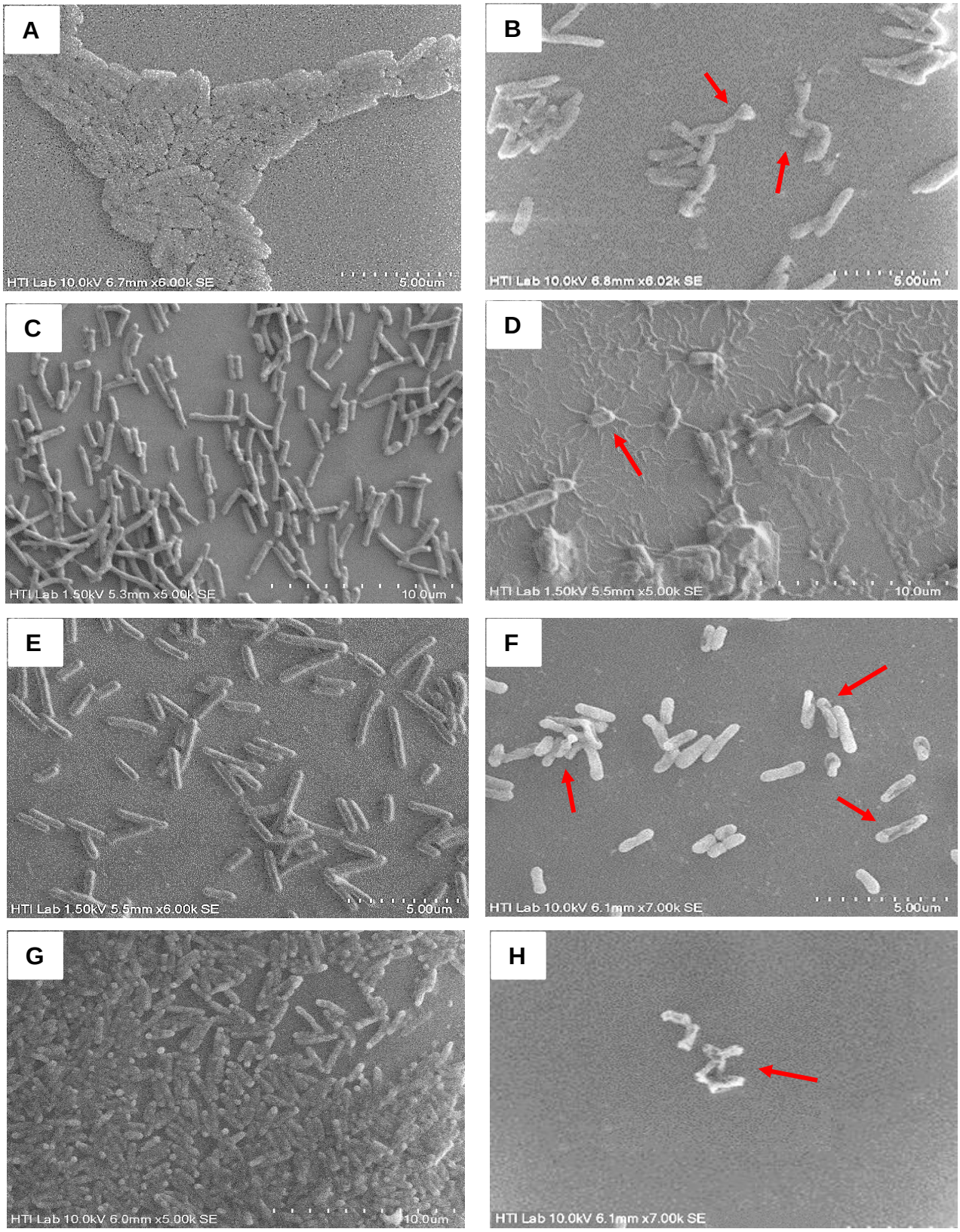

Figure 1: Comparison between untreated and treated bacteria with *P. macrocarpa* extract. (A) Morphology of untreated *Pseudomonas mallei*. (B) Morphological alterations of *Pseudomonas mallei* treated with extract. (C) Morphology of untreated *E.coli*. (D) Morphological alterations of *E.coli* treated with extract. (E) Morphology of untreated *Klebsiella pneumonia*e. (F) Morphological alterations of *Klebsiella pneumonia*e treated with extract. (G) Morphology of untreated *Klebsiella aerogenes*. (H) Morphological alterations of *Klebsiella aerogenes* treated with extract. *(The abnormalities of bacterial cells is indicated by the red arrow).*

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