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# Antiangiogenic activity of sophorolipids extracted from refined bleached deodorized palm olein

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## Abstract:

**OBJECTIVES:** Sophorolipids (SLs) are a group of surface-active glycolipids produced by a type of nonpathogenic yeast *Candida bombicola* in the presence of vegetable oil through fermentation technology. SLs have shown antitumor activity; however, the mechanism of action underlying the anticancer activity of SLs is poorly understood. This work evaluated the anticancer activity of SLs fermented from palm oil by exploring its antiangiogenic activity.

**MATERIALS AND METHODS:** The SLs that were fermented and further characterized for their biochemical activities. Cytotoxicity study was performed to assess cytostatic properties. A series of *in vitro* and *ex vivo* angiogenesis assay was also carried out. The relative fold change in the expression of p53 mRNA by SLs was also studied.

**RESULTS:** Altogether, the data show that SLs derived from palm oil fermentation process inhibited neovascularization in the *ex vivo* tissue segments and also the endothelial cell proliferation between 50% and 65% inhibition as a whole. The palm oil derived SLs also caused downregulation of the suppression level of vascular endothelial growth factor and also upregulate the p53 mRNA level. The analytical studies revealed the presence of high amount of phenolic compounds but with relatively weak antioxidant activity. The gas chromatography-mass spectrometry studies revealed abundant amount of palmitic and oleic acid, the latter an established antiangiogenic agent, and the former being proangiogenic.

**CONCLUSION:** Therefore, it can be concluded from this study that SLs derived from fermented palm oil have potent antiangiogenic activity which may be attributed by its oleic acid component.

## Keywords:

Antiangiogenic, anticancer, fermentation, palm oil, sophorolipids

## Introduction

Angiogenesis means the growth of new blood vessels. It is an important natural process occurring in the body both in healthy and diseased conditions. Angiogenesis occurs in the healthy body for healing wounds and for restoring blood flow to tissues after injury or insult. It is also an essential process in the growth and spread of

cancer. Tumors grow and spread massively with the aid of continuous blood supply.

Biosurfactants are surface-active metabolites synthesized biologically by microorganisms. Biosurfactants are widely used in many industries because they are unique.<sup>[1]</sup> Most yeast biosurfactants have been typically identified as glycolipids. The most interesting glycolipids are sophorolipids (SLs) because SLs are biodegradable, possess low ecotoxicity, and are also producible in high yield. SLs can be synthesized fermentatively

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only by a selected number on yeast species as they are known to be surface-active compounds.<sup>[2]</sup> The nonpathogenic yeast *Candida bombicola* used in this fermentation process to produce SLs. *C. bombicola* uses glucose as the primary carbon source converting the fermentation medium and oil into SLs.

SLs are of growing commercial interest because they can be easily produced in extremely high yields from cheap raw materials, such as vegetable oils, n-alkanes, and industrial waste product.<sup>[2]</sup>

Palm oil-derived SLs have been demonstrated to have antioxidant properties, and some studies have shown that SLs have potent anticancer activity.<sup>[3]</sup>

## Materials and Methods

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Affymetrix, USA. *C. bombicola* 22214 and EA.hy926 were obtained from ATCC, USA. Calbiochem, USA, and Corning, USA, supplied fibrinogen and matrigel, respectively. Dulbecco's Modified Eagle's Medium Powder and Medium 199 were purchased from Gibco, Life Technology, UK. Yeast extract and yeast malt agar were purchased from HiMedia, India. Ascorbic acid, Folin-Ciocalteu reagent, gallic acid, hydrochloric acid, and methanol were acquired from Merck, Germany. Solvents were purchased from QReC, New Zealand. Raybio, USA supplied human vascular endothelial growth factor (VEGF) Assay Kit. Acetic acid, ferrous sulfate, sodium acetate trihydrate, and sodium carbonate were obtained from R and M Chemicals, Essex, UK. 2,2'-azinobis (3-ethylbenzthiazolinesulfonic acid), 2,4,6-tris(1-pyridyl)-5-triazine (TPTZ), aprotinin, CaCl<sub>2</sub>, C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>, crystal violet, 1, 1-diphenyl-2-picrylhydrazyl (DPPH),  $\epsilon$ -aminocaproic acid, KH<sub>2</sub>PO<sub>4</sub>, L-glutamine, MgSO<sub>4</sub>.7H<sub>2</sub>O, Na<sub>2</sub>CO<sub>3</sub>, NaCl, Na<sub>2</sub>SO<sub>4</sub>, potassium persulfate, suramin, and thrombin 1000 unit were purchased from Sigma-Aldrich, Germany.

### Fermentation and recovery of sophorolipids

Cultivation studies were carried out in a 1 L benchtop bioreactor (BioTron, Korea) with stirring rate of 400 rpm, pH at 5.5, and the run air to 1 v/v. Seed medium consisting of 10% glucose, 0.5% yeast extract, and mineral salt was prepared and incorporated into the bioreactor vessel. The medium used contained (in g/L) yeast extract, 5.0; Mg SO<sub>4</sub>.7H<sub>2</sub>O, 5.0; KH<sub>2</sub>PO<sub>4</sub>, 1.0; CaCl<sub>2</sub>, 0.1; and NaCl, 0.1. 800 mL of cultivation medium, excluding glucose, was incorporated into the fermenter vessel. The pH of the medium in vessel was adjusted to 5.5. The bioreactor was let to run for 2 days. On day 2, palm oil was added into the fermenter vessel in a sterile condition. The fermentation process was stopped on day 6.

Ethyl acetate and n-hexane were used to separate crude SLs from the fermentation product. The end product was dried overnight in an oven at 105 °C to completely remove n-hexane and dried again in desiccator over anhydrous Na<sub>2</sub>SO<sub>4</sub> for 48 h before weighing.

### Ultraviolet visible spectrophotometry

Ultraviolet (UV) spectrum of SLs was recorded using Lambda 45 UV/Vis Spectrophotometer system (Perkin-Elmer, USA) and screened on spectra range of 500–200 nm.

### Fourier transform-infrared spectrometry

The infrared (IR) spectra were obtained on Fourier transform-IR (FT-IR) spectrometer (Thermo. Nicolet Nexus 670, Thermo Scientific, USA) system connected to OMNIC software (Thermo, Electron Corporation, USA). The spectra were measured within the range of 400–4000cm<sup>-1</sup>.

### Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) (Agilent 6890N/5973I, USA) equipped with electrospray ionization was used. About 1  $\mu$ L of sample (10 mg/mL) was injected. A nonpolar HP-5MS capillary column (0.25 mm  $\times$  30 m  $\times$  0.25  $\mu$ m film thickness) packaged with stationary phase and equipped with quadrupole detector was used. The temperature was increased to 305°C at a rate of 20°C/min. The compound mass ranged between 35 and 700 m/z at a scanning rate of 1 scan/second, and mass spectra obtained were compared to the database from the National Institute of Standard and Technology 02 library. MSD ChemStation Data were connected to the system for data analysis.

### Estimation of total phenolic content

Folin-Ciocalteu reagent, sodium carbonate, SLs, and distilled water were mixed in a test tube in a ratio of 5:15:1:79 to the final volume of 1000  $\mu$ L. All the tubes were incubated for 2 h at room temperature. Then, 200  $\mu$ L of each mixture was added in a 96-well microplate.<sup>[4]</sup> Distilled water was used as blank. The absorbance was recorded at 765 nm, and the results expressed as  $\mu$ g gallic acid/equivalent/mg dry extract.

### 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) scavenging activity

14 mmol/L of ABTS and 4.9 mmol/L of potassium persulfate of equal volumes were dissolved in deionized water and react in the dark at 25 °C for 20 h to form the radical cation solution of (ABTS<sup>•+</sup>). The solution was diluted and read at 734 nm. Ascorbic acid was used as reference standard. The results were expressed as IC<sub>50</sub>.

### 1, 1-diphenyl-2-picrylhydrazyl free radical scavenging assay

About 100  $\mu\text{L}$  of each sample (at six concentrations) was mixed with 100  $\mu\text{L}$  of DPPH dissolved in methanol (200  $\mu\text{mol/L}$ ) and incubated in a dark place at 25  $^{\circ}\text{C}$  for 30 min. The remaining amount of DPPH was determined at 517 nm. Ascorbic acid was used as reference standard.

### Ferric reducing antioxidant power assay

150  $\mu\text{L}$  ferric reducing antioxidant power (FRAP) solution (300 mmol/L acetate buffer, pH 3.6, 10 mmol/L TPTZ in 40 mmol/L HCl and 20 mmol/L  $\text{FeCl}_3$  in a ratio 10:1:1) was added to 50  $\mu\text{L}$  of each sample and incubated at room temperature for 8 min and read the absorbance at 600 nm. Ferrous sulfate was used as reference standard. All results were expressed as  $\text{Fe}^{+2}$  equivalent/mg dry extract.

### 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide cell viability assay

EA.hy926 cells were treated with SLs and incubated at 37 $^{\circ}\text{C}$  with 5%  $\text{CO}_2$  in humid atmosphere for 48 h. The solubilized formazan product was quantified using TECAN Microplate Reader to calculate the percentage inhibition of cell proliferation.<sup>[5]</sup>

### Rat aorta ring assay

Thoracic aortas were removed from euthanized rats. The aortas were cross sectioned into small rings (approximately 1-mm thickness) and seeded individually in 48-wells plate in 300  $\mu\text{L}$  serum-free M199 media containing 3 mg/mL fibrinogen and 5 mg/mL aprotinin. Ten microliters of thrombin (50 NIH U/mL in 1% bovine serum albumin in 0.15 M NaCl) were added into each well and incubated at 37 $^{\circ}\text{C}$  for 90 min to be solidified. A second layer (M199 medium supplemented with 20% HIFBS, 0.1%  $\epsilon$ -aminocaproic acid, 1% L-glutamine, 2.5  $\mu\text{g/mL}$  amphotericin B, and 60  $\mu\text{g/mL}$  gentamicin) was added into each well (300  $\mu\text{L/well}$ ).<sup>[6]</sup> On day 5, aortic rings were photographed using an inverted microscope ( $\times 40$ ), and subsequently, the length of blood vessels outgrowth from the primary tissue explants was measured using ImageJ software. All experimental procedures were conducted following the Animal Ethics Guidelines of Universiti Sains Malaysia (USM/Animal Ethics Approval/2015/[97] [713]).

### Colony formation assay

EA.hy926 cells were incubated for 12 h to facilitate their attachment. Colonies formed were then fixed with 4% paraformaldehyde and stained with 0.2% (w/v) crystal violet. The colonies were counted under a stereomicroscope.

### Cell migration assay

EA.hy926 cell line was treated with SLs at different concentrations and photographed at 0, 6, and 18 h using an inverted microscope. The migrated area was measured using ImageJ software to calculate the percentage inhibition of wound closure.<sup>[7]</sup>

### Tube formation assay

EA.hy926 cells were treated with concentrations of SLs and incubated at 37 $^{\circ}\text{C}$  for 24 h were added to 48-well plates and allowed to polymerize in incubator for 45 min at 37 $^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . The cells were then harvested and seeded onto the matrigel-coated plates. The cells were imaged using an inverted microscope at low magnification and the network length and width were quantified using Wimasis software (Wimasis GmbH Munich, Germany) to measure the percentage inhibition of tube formation.

### Vascular endothelial growth factor expression

The VEGF concentration in EA.hy926 cells was determined using a human VEGF-165 ELISA kit (Raybio, USA).

### p53 transcription factor expression

p53 transcription factor expression of SLs was measured using Signal<sup>TM</sup> Finder reporter array system. 50  $\mu\text{L}$  of the transfection reagent (FuGENE<sup>®</sup>, Promega, USA), prepared to 6  $\mu\text{L}/\mu\text{g}$  DNA in Opti-MEM medium, was added into each well of the array plate. The reaction mixture was incubated at room temperature for 20 min. EA.hy926 cells ( $5 \times 10^4/\text{mL}$ ) were resuspended in Opti-MEM medium containing 10% FBS and 1% nonessential amino acids without antibiotics. Cells were observed under green filter under fluorescent microscope (EVOS *fl*, Digital microscopy group, USA). The transfection efficiency was calculated.

Dual-Glo luciferase reporter system (Promega, USA) was used to measure the luciferase signals for each pathway in control and treated cells. The results are presented as mean  $\pm$  standard deviation (SD) of relative fold change in the activity of each pathway ( $n = 2$ ).

Relative fold change in activity

$$= \left( \frac{\text{FR ratio of treatment group}}{\text{FR ratio of control group}} \right)$$

### Statistical analysis

Results are presented as mean  $\pm$  SD of three independent experiments ( $n = 3$ ). One-way ANOVA, followed by *post hoc* tukey was executed using GraphPad Prism (San Diego, CA, USA) software.

## Results

### Fermentation and recovery of sophorolipids

A volume of 3 L of solvents was used to recover 1 L of SLs obtained at the end of fermentation. Total SLs yield obtained after recovery was 57.5 g/L. The SLs acquired appeared to be brown-colored oil. The appearance changes to yellowish solid at room temperature. The pH of the fermentation end product was 5.5.

### Ultraviolet-visible spectrophotometry

Figure 1 shows the UV absorption spectrum of SLs. The spectra showed strong absorption at  $\lambda_{\max}$  200–210 nm. Besides, SLs showed obvious peaks between  $\lambda_{\max}$  220 and 290 nm.

### Fourier transform-infrared spectrometry

Figure 2 demonstrates FT-IR spectrum of SLs with wave number ranging from 1000 to 3000  $\text{cm}^{-1}$ . FT-IR spectrum demonstrates three sharp vibrational bands present at 2922.06, 2850.22, and 1740.64  $\text{cm}^{-1}$ , indicating the presence of C-H and C=O stretching which belongs to alcohol and aldehyde groups, respectively.

### Gas chromatography-mass spectrometry

The GC-MS analysis of SLs demonstrates the presence of fatty acid in vast amount as depicted in Figure 3. Six compounds were found to have similarity more than 90% in SLs which are myristic acid, pentadecyclic acid, palmitic acid, oleic acid, and ethyl oleate. Among these six compounds, palmitic acid, followed by oleic acid was found to be present in huge amount (60.28% and 25.71%, respectively).

### Antioxidant assays

Table 1 shows the result obtained from different antioxidant assays of SLs. Based on Table 1, SLs exhibited weak antioxidant capacity.

### 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide cell viability assay

After 48 h of treatment, no significant difference was observed at concentrations 6, 12, and 25  $\mu\text{g}/\text{mL}$  of SLs ( $P > 0.05$ ). This shows that there is no sign of toxicity at these three doses. The  $\text{IC}_{50}$  value of SLs on EA.hy926 cells was  $110.49 \pm 12.05 \mu\text{g}/\text{mL}$  which shows the noncytotoxic nature of SLs toward the cells [Figure 4].

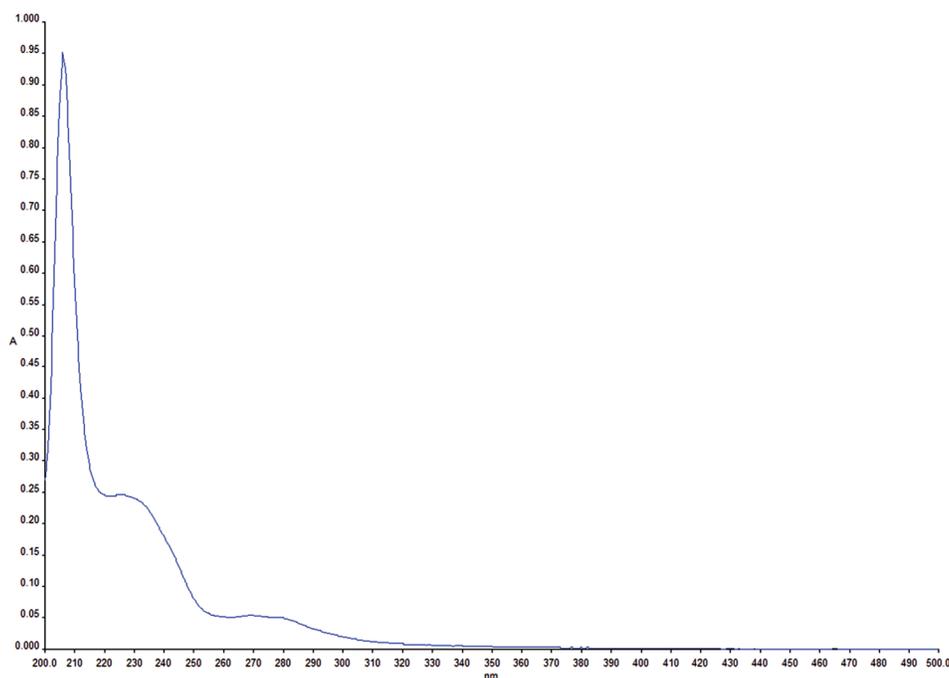


Figure 1: Ultraviolet-Vis absorption spectrum of sophorolipids

Table 1: Total phenolic contents and antioxidant values of sophorolipids

Samples	TPC ( $\mu\text{g}$ gallic acid equivalent/mg extract)	ABTS ( $\mu\text{g}/\text{mL}$ )	DPPH ( $\mu\text{g}/\text{mL}$ )	FRAP (nmol $\text{Fe}^{+2}$ equivalent/mg extract)
SLs	$7.57 \pm 0.30$	$473.24 \pm 34.87$	$487.64 \pm 1.95$	$64.67 \pm 0.25$
Reference (ascorbic acid)	-	$3.58 \pm 0.05$	$0.98 \pm 0.02$	-

TPC=Total phenolic content, SLs=Sophorolipids, DPPH=1, 1-diphenyl-2-picrylhydrazyl, FRAP=Ferric reducing antioxidant power, ABTS=2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid

### Rat aorta ring assay

Figure 5 shows the antiangiogenic activity of SLs on rat aorta rings. At concentrations 6, 12, 25, 50, and 100  $\mu\text{g}/\text{mL}$  of SLs and suramin at 100  $\mu\text{g}/\text{mL}$ , the percentage inhibition of blood vessel outgrowth was  $19.57\% \pm 13.09\%$ ,  $28.65\% \pm 10.45\%$ ,  $33.69\% \pm 5.98\%$ ,  $46.01\% \pm 12.34\%$ ,  $64.87\% \pm 10.50\%$ , and  $71.14\% \pm 7.46\%$ , respectively [Figure 6]. There were no significant activities when the data of treatments at 6, 12, and 25  $\mu\text{g}/\text{mL}$  of SLs were compared among each other ( $P > 0.05$ ). At 50  $\mu\text{g}/\text{mL}$ , there were significant activities as compared to activities of treatment at lower doses ( $P < 0.001$ ).

### Colony formation assay

Figure 7 shows the potency of SLs to inhibit the colony formation of EA.hy926 cells. The percentage

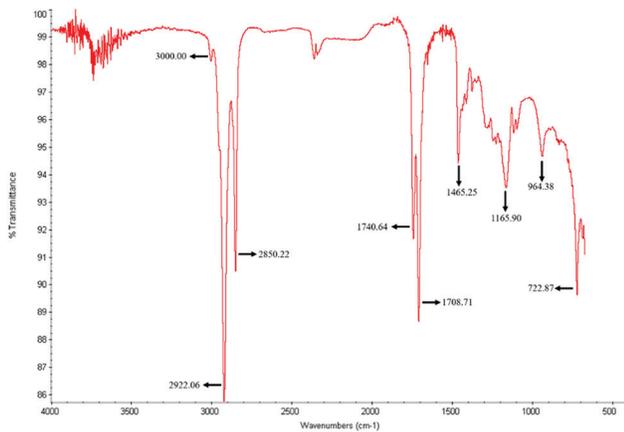


Figure 2: Fourier transform-infrared spectrum of sophorolipids illustrating the presence of major functional group of sophorolipids

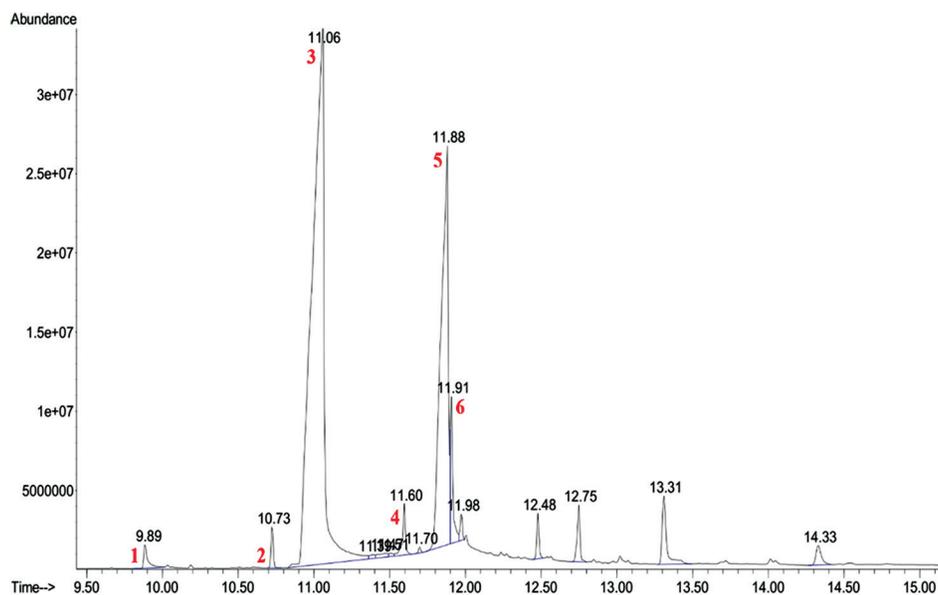


Figure 3: The gas chromatography-mass spectrometry chromatogram of sophorolipids

inhibition of colony formation at concentrations 50  $\mu\text{g}/\text{mL}$ , 100  $\mu\text{g}/\text{mL}$ , and 100  $\mu\text{g}/\text{mL}$  of suramin was  $45.70\% \pm 1.13\%$ ,  $54.60\% \pm 2.51\%$ , and  $65.60\% \pm 2.65\%$ , respectively [Figure 8]. Figure 7 (a) illustrates significant difference when the inhibitory activity of 50  $\mu\text{g}/\text{mL}$  SLs was compared to suramin as positive control ( $P < 0.001$ ).

### Cell migration assay

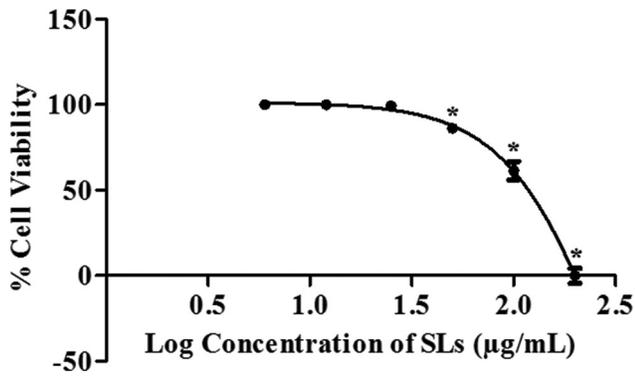
Figure 9 shows the antimigratory activity of SLs on EA.hy926 cells at different treatment periods. The wound created in 0.5% DMSO group was closed after 18 h of treatment. The percentage of wound closure at 6 h in 0.5% DMSO, treatment groups (50 and 100  $\mu\text{g}/\text{mL}$  of SLs), and 100  $\mu\text{g}/\text{mL}$  of suramin was  $30.43\% \pm 6.63\%$ ,  $14.60\% \pm 6.59\%$ ,  $9.56\% \pm 5.82\%$ , and  $47.00\% \pm 6.50\%$ , respectively [Figure 10]. After 6 h of treatment, the inhibitory activity of 50  $\mu\text{g}/\text{mL}$  SLs was compared with the control group, and it showed significantly moderate activity ( $P < 0.01$ ).

### Tube formation assay

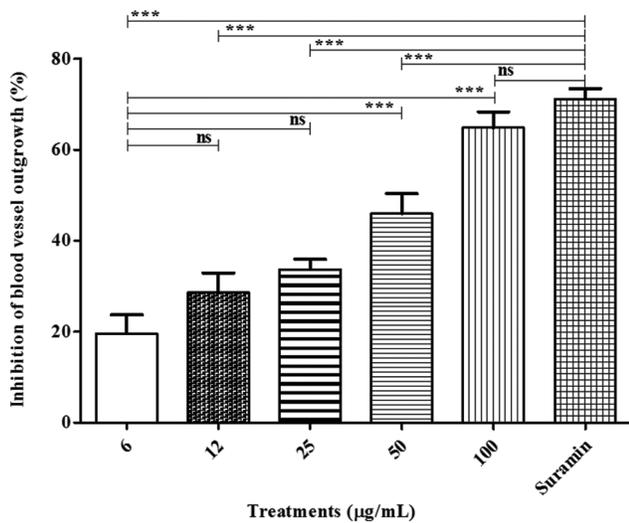
Figure 11 illustrates the tendency of SLs to inhibit EA.hy926 tube formation process. The percentage inhibition of tube formation at 50  $\mu\text{g}/\text{mL}$  of SLs, 100  $\mu\text{g}/\text{mL}$  of SLs, and 100  $\mu\text{g}/\text{mL}$  of suramin was  $47.40\% \pm 19.20\%$ ,  $52.20\% \pm 14.90\%$ , and  $76.70\% \pm 4.38\%$ , respectively [Figure 12]. Based on Figure 12, the inhibitory activity of 100  $\mu\text{g}/\text{mL}$  of SLs was almost the same as compared to suramin as positive control in equivalent concentration ( $P > 0.05$ ).

### Determination of vascular endothelial growth factor

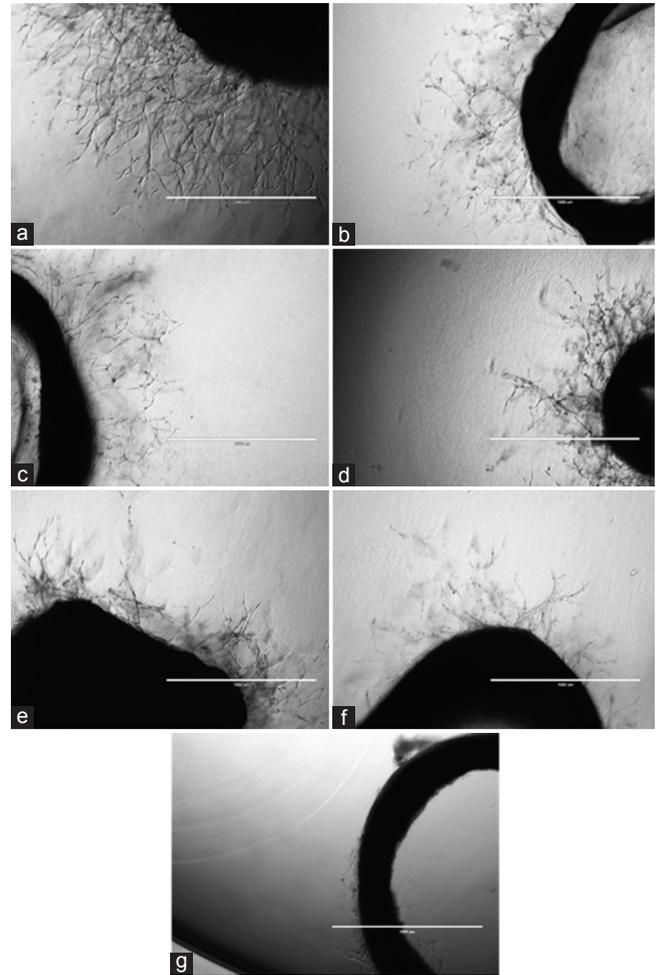
Figure 13 highlights the amount of VEGF secreted by EA.hy926 cells in 0.5% DMSO, 50 and 100  $\mu\text{g}/\text{mL}$  of



**Figure 4:** Effect of sophorolipids extract on cell viability of EA.hy926 cells after 48 hours of exposure to different concentrations. Values shown mean  $\pm$  SD of three independent experiments ( $n = 3$ ). \* $P < 0.05$ . SD = Standard deviation



**Figure 6:** Dose-response relationship of different concentrations of SLs (6–100 µg/mL) against suramin (positive control). Values are presented as mean  $\pm$  SD ( $n = 3$ ). \*\*\* $P < 0.001$  and ns denotes  $P > 0.05$ . SLs = Sophorolipids, SD = Standard deviation, ns = Not significant

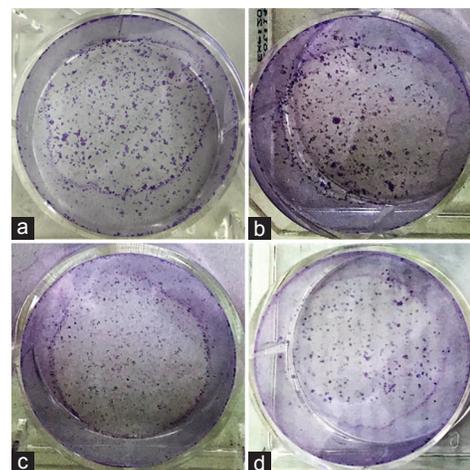


**Figure 5:** The antiangiogenic activity of SLs on blood vessel outgrowth in *ex vivo* rat aorta rings. The rings were treated with (a) 0.5% DMSO, (b) 6 µg/mL of SLs, (c) 12 µg/mL of SLs, (d) 25 µg/mL of SLs, (e) 50 µg/mL of SLs, (f) 100 µg/mL of SLs, and (g) 100 µg/mL of suramin as positive control. SLs = Sophorolipids, SD = Standard deviation, DMSO = Dimethyl sulfoxide, ns = Not significant

SLs and the percentage inhibition of VEGF release in the respective treatment groups. The VEGF concentrations released in 0.5% DMSO and 50 and 100 µg/mL of SLs were  $71.40 \pm 9.52$ ,  $45.60 \pm 4.02$ , and  $38.60 \pm 2.49$  pg/mL, respectively. There was a significant difference when the activities of 50 and 100 µg/mL treatment groups were compared to the control group ( $P < 0.01$ ), whereas there was no statistical difference when the comparison was made between the treated groups ( $P > 0.05$ ).

### p53 transcription factor expression

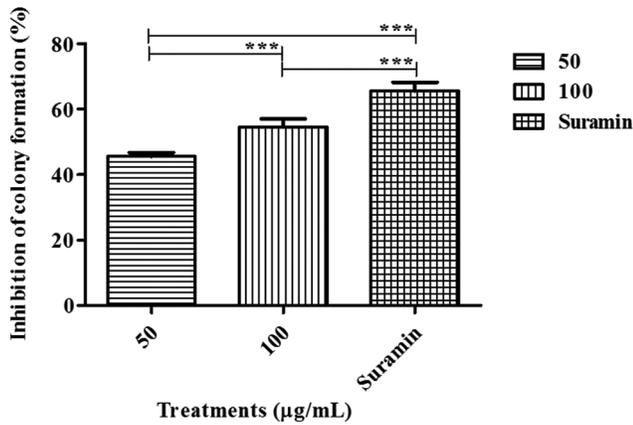
Figure 14 shows the activity of SLs on the p53 transcription factor expression level. The relative fold change in p53 activity was  $2.15 \pm 0.34$ . Relative fold change value more than 1 designates the upregulation of p53 transcription factor. The activity of SLs at 100 µg/mL indicated significant difference by upregulating the activity of p53 transcription factors ( $P < 0.05$ ).



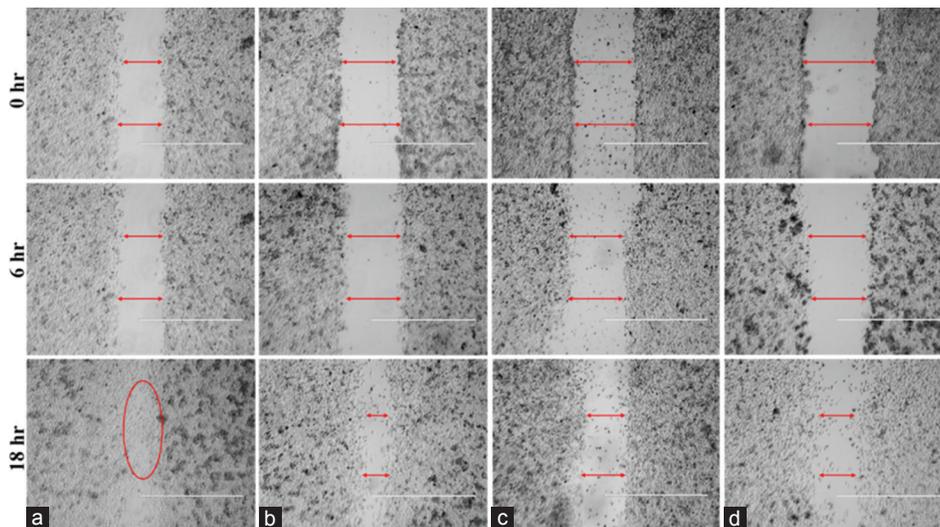
**Figure 7:** Effect of SLs treatment on clonogenic potential of EA.hy926 cells at concentrations (a) 0.5% DMSO, (b) 50 µg/mL of SLs, (c) 100 µg/mL of SLs, and (d) suramin (100 µg/mL). SLs = Sophorolipids, DMSO = Dimethyl sulfoxide

## Discussion

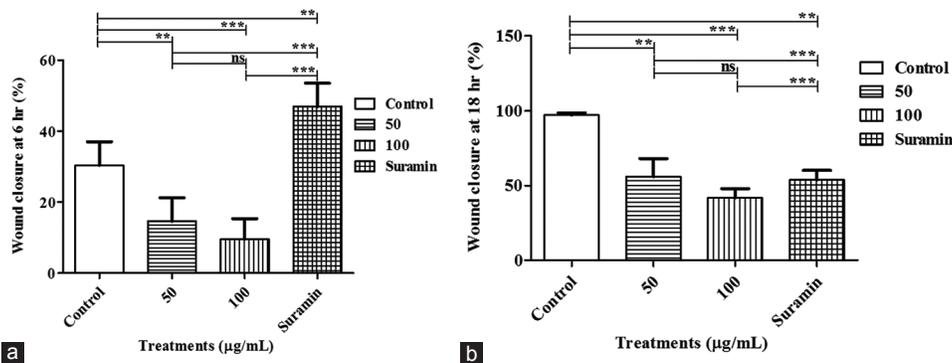
Palm oil was used in this study as it is readily available in Malaysia and free from impurities, which promote a protected application, particularly toward consumer well-being. SLs were isolated from the nonpathogenic yeast *C. bombicola*. The end product obtained after the 6-day fermentation process was a mixture of acidic and lactonic SLs. In the present study, the fermentation broth did not appear to be foaming; hence, it can be concluded that lactonic SLs might be present in excess as compared to acidic moiety.<sup>[8]</sup> In general, fatty acids consist of long hydrocarbon chain linked to a carboxyl group (COOH). Fatty acid which comprises 60% of SLs is palmitic acid. Palmitic acids are made up of saturated fats;<sup>[9]</sup> hence, they appear to be solid at room temperature. On the other hand, SLs contain 25% of oleic acid which is known to be unsaturated fats.<sup>[10]</sup>



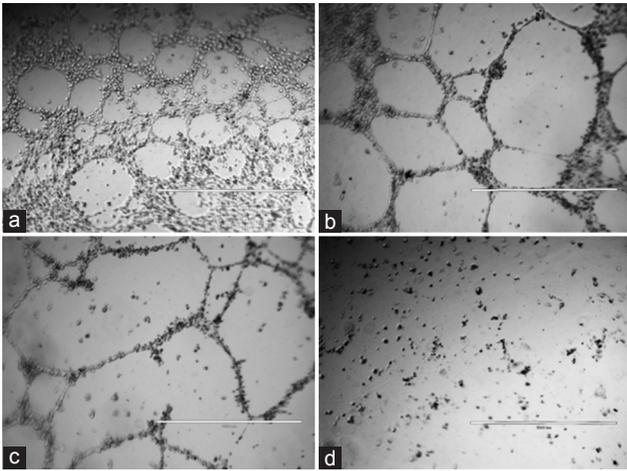
**Figure 8:** Graphical representation of percentage inhibition of colony at various treated concentrations. Control = 0.5% DMSO, 50 = 50 µg/mL of SLs, C = 100 µg/mL of SLs, and suramin = 100 µg/mL of suramin. Values are presented as mean ± SD (n = 3). \*\*\*P < 0.001. SLs = Sophorolipids, SD = Standard deviation, DMSO = Dimethyl sulfoxide



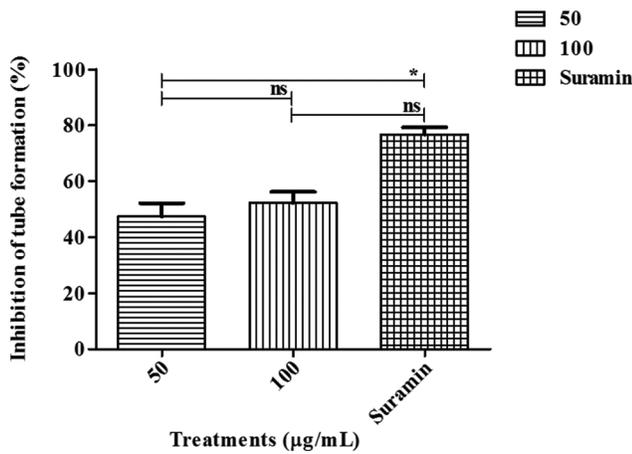
**Figure 9:** Effect of SLs on inhibition of EA.hy926 cell migration at concentrations (a) 0.5% DMSO, (b) 50 µg/mL of SLs, (c) 100 µg/mL of SLs, and (d) suramin (100 µg/mL) at 0, 6, and 18 h. Photos were pictured at ×4 (1000 µm scale bar). SLs = Sophorolipids, DMSO = Dimethyl sulfoxide



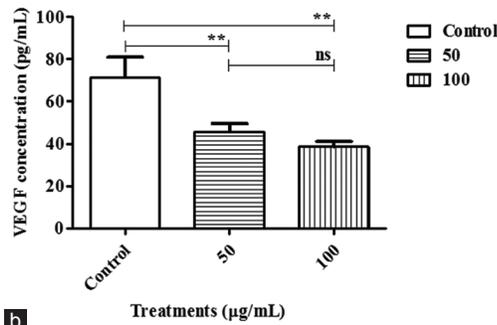
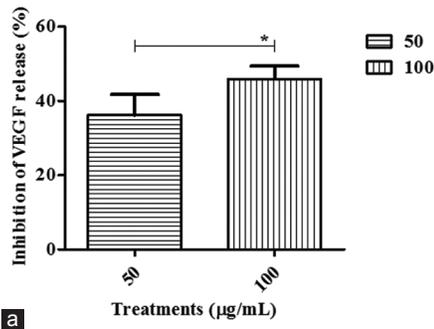
**Figure 10:** (a) Graphical representation of percentage of wound closure at 6 h, (b) Graphical representation of percentage of wound closure at 18 h. Control = 0.5% DMSO, 50 = 50 µg/mL of SLs, 100 = 100 µg/mL of SLs, and suramin = 100 µg/mL of suramin. Values are presented as mean ± SD (n = 3). \*\*\*P < 0.001, \*\*P < 0.01, and ns denotes P > 0.05. DMSO=Dimethyl sulfoxide, SLs=Sophorolipids, SD=Standard deviation, ns=Not significant



**Figure 11:** Antiangiogenic activity of SLs on EA.hy926 cell tube formation at concentrations (a) 0.5% DMSO, (b) 50 µg/mL of SLs, (c) 100 µg/mL of SLs, and (d) suramin (100 µg/mL). Tubes were photographed at × 4 (scale bar 1000 µm). DMSO = Dimethyl sulfoxide, SLs = Sophorolipids



**Figure 12:** Graphical representation of percentage inhibition of tube formation. Control = 0.5% DMSO, 50 = 50 µg/mL of SLs, 100 = 100 µg/mL of SLs, and suramin = 100 µg/mL of suramin. Values are presented as mean ± SD (*n* = 3). \**P* < 0.05 and ns denotes *P* > 0.05. SLs = Sophorolipids, DMSO = Dimethyl sulfoxide, SD = Standard deviation, ns = Not significant



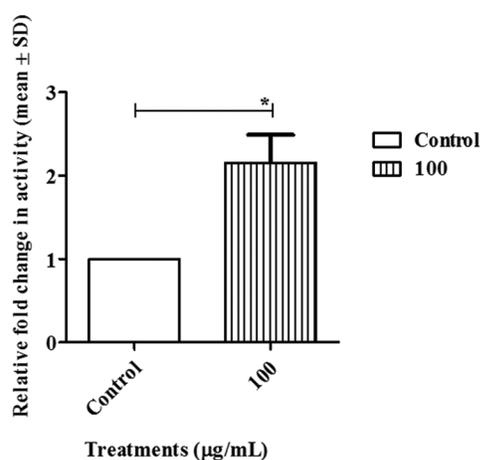
**Figure 13:** (a) Activity of SLs on the release of VEGF-A from EA.hy926 cells. Control = 0.5% DMSO and 100 = 100 µg/mL of SLs. (b) Concentration of VEGF-A from EA.hy926 cells. Control = 0.5% DMSO and 100 = 100 µg/mL of SLs. Values shown are presented as mean ± SD (*n* = 3). \*\**P* < 0.01, \**P* < 0.05, and ns denotes *P* > 0.05. SLs = Sophorolipids, SD = Standard deviation, VEGF = Vascular endothelial growth factor, ns = Not significant

The result of FT-IR analysis matches the peaks obtained, which was reported in previous study of SLs. The maximum UV absorption of SLs in the present study, which is within  $\lambda_{\max}$  200–210 nm, matches the outcome obtained in previous studies which have been used for high-performance liquid chromatography analysis.<sup>[11]</sup> SLs were subjected to GC-MS analysis to investigate the type of compounds present in the extract. The presence of six compounds based on GC-MS data were fatty acids. Among all the antioxidant assays conducted together with estimation of total phenolic content, FRAP assay is a major contributor toward the antioxidant activity of SLs. On the other hand, phenolic content presents in SLs, ABTS and DPPH assays did not give rise to the antioxidant capacity of SLs, although they could accelerate SLs' activity at the least.

The result obtained from antiangiogenic activity of SLs using rat aorta ring assay demonstrated significant inhibition of the sprouting blood vessels with an  $IC_{50}$  of 63.89 µg/mL. Hence, the findings from MTT assay and rat aorta ring assay on EA.hy926 cells reveal the antiangiogenic activity of SLs. Since SLs did not show any cytotoxic effect toward the cell, the antiangiogenic property could be mainly due to the inhibition property of SLs in the first stage of angiogenesis process. Colony formation assay results also confirmed the MTT assay results.

SLs in migration assay would have possibly inhibited the cell migration to prevent their penetration by downregulating VEGF.<sup>[12]</sup> SLs in the tube formation study inhibited the tube formation of endothelial cells by 52% which is almost comparable to the percentage inhibition obtained from previous assays.<sup>[13]</sup>

VEGF plays a major role in the angiogenesis process. An extensive range of living organisms' tissues express VEGF at a very low level. However, VEGF is secreted



**Figure 14:** Activity of SLs on the expression of p53 transcription factor. Control = 0.5% DMSO and 100 = 100 µg/mL of SLs. Values are presented as mean ± SD ( $n = 2$ ). \* $P < 0.05$ . SLs = Sophorolipids, SD = Standard deviation, DMSO = Dimethyl sulfoxide

at high levels when required; for instance, during the development of embryo and in human tumors. In the present research, there was a dose-dependent trend observed in the release of VEGF as well as the percentage inhibition of the release. Therefore, SLs could possibly contribute toward its antiangiogenic property. It can be suggested that SLs have the capability to downregulate VEGF, especially VEGF-A release which in turn hinders the activation of EA.hy926 cells. Inactivation of the endothelial cells discontinues the cell colony formation, cell migration, and tube formation process.<sup>[14]</sup> These multistep processes finally slowdown the outgrowth of new blood vessels, which are demonstrated in the rat aorta ring assay.<sup>[15]</sup> In a recent study, a team of researcher has also demonstrated that some fatty acids can inhibit the angiogenesis process by downregulation of VEGF.<sup>[16]</sup>

p53 is a tumor suppressor protein which plays a major role in the angiogenesis process.<sup>[17]</sup> P53 may modulate the VEGF expression besides stimulating proteins to repair damaged DNA as an anticancer agent.<sup>[18]</sup>

Based on p53 fold changes obtained in the present research, it can be said that SLs might have upregulated the expression of p53 mRNA level toward antiangiogenic pathway and conclude in tumor inhibition. Overall, SLs upregulated p53 expression level which downregulated VEGF expression causing the inhibition of angiogenesis process.

## Conclusion

Biosurfactants, especially glycolipid, are gaining more commercial interest due to its unique properties. This study demonstrated that SLs have yellowish texture in room temperature with PH of 5.5. FT-IR and UV-vis results confirmed the presence of alcoholic and aldehyde

groups in the final product of fermented SLs. The majority of the compounds in SLs end product was revealed to be palmitic acid and oleic acid based on GC-MS analysis. Even though SLs did not demonstrate cytotoxic and significantly antioxidant effect, but it showed significantly antiangiogenic effect *in vitro* and *ex vivo* compared with suramin as approved drug. Moreover, downregulation of VEGF release and upregulation of P53 transcription factors confirmed the *in vitro* and *ex vivo* antiangiogenic effect of SLs.

Based on the results obtained, it can be concluded that SLs have promising potential to target cancer angiogenesis.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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