NOTE:

Synthesis, Sunscreen, and Toxicity *In Vitro* Test of C-Styrylcalix[4]resorcinaryl Octacinnamate and C-Phenylcalix[4]resorcinaryl Dodecacinnamate

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Abstract: *The need for qualified sunscreen materials has increased from year to year. This prompt researchers to find new sunscreen ingredients that have good activity. In this study, new C-styrylcalix[4]resorcinaryl octacinnamate* ***1*** *and C-phenylcalix[4]resorcinaryl dodecacinnamate* ***2*** *have been synthesized via esterification reaction. The target molecules were characterized by FTIR,* ^{1}*H-NMR and LC-MS spectrometers. The sunscreen activity was evaluated using an ultraviolet spectrophotometer and the cytotoxicity assay was tested on kidney Vero cells using the cell culture method. Compound* ***1*** *was obtained as light brown solid in 68% yield with the melting point of 247 °C. Compound* ***2*** *was obtained as light yellow solid in 69% yield with melting point of 268°C. The sunscreen test shows that* ***1*** *and* ***2*** *can absorb UV-B radiation with the SPF values of 67.45 and 70.85, respectively. The cytotoxicity assay shows that the* IC_{50} *values of* ***1****,* ***2****, and parasol are 1468.2, 676.1 and 758.7 µg/mL, respectively. Based on the sunscreen activity test and toxicity assay, it can be said that calix[4]resorcinarenes* ***1*** *and* ***2*** *have potential to be developed as sunscreen ingredients.*

Keywords: *synthesis; sunscreen; toxicity; esterification*

■ INTRODUCTION

The cases of skin cancer have increased from year to year, where the estimated 132,000 cases of melanoma skin cancer occurred each year. The parts of body often affected by skin cancer are the ones that are most frequently exposed to sunlight, such as the face as well as skin of the face and hands [1]. The types of skin damage due to sun exposure are erythema, edema, sunburn, tanning, hyperplasia, immunosuppression, photoaging, and skin cancer [2-4]. The factors that cause the widespread of the cases of skin cancer are the chronic exposure to sunlight, climatic changes, and individual as well as social conditions [5-7]. The skin damage can be minimized by applying the sunscreen. Sunscreen cream is a product that has a formula working to absorb, to scatter or to reflect ultraviolet light and to minimize the intensity of ultraviolet rays that directly hit the skin [8-9].

Some of the requirements of a sunscreen products are safe to the body parts that are smeared, high ultraviolet absorbance over a wide range of wavelengths, stable towards the ultraviolet light, easy to prepare and economically profitable [10-11]. The action mechanism of skin care products is to absorb or to reflect ultraviolet A or B rays, thereby reducing the cell degradation of reactive oxygen species (ROS) that cause the photoaging [12]. The solar ultraviolet radiations (UVR) are divided into three categories according to their wavelength namely ultraviolet-C (200–280 nm), ultraviolet-B (280–320 nm), and ultraviolet-A (320–400 nm) [11]. Ultraviolet-C is the most biologically damaging radiation, but it is filtered out by the ozone layer. Among the solar radiations, UV-B ray are the most influential to the human skin health since UV-B rays can pass through the ozone layer [12]. Reactive oxygen species (ROS) or reactive nitrogen species (RNS) generated due to the

interaction between UV-B rays and the skin may cause inflammation, sunburn, premature aging, and even cancer [11,13-14].

Various attempts have been conducted to develop new sunscreen compounds, one of which is the synthesis of sunscreen compounds by modifying the structure of compounds known to display high sunscreen activity. The structural modifications or preparation of analog compounds are mostly investigated since they are cheaper and faster to carry out. The *in vitro* assay can be carried out through the ultraviolet spectrometry method and can also use the human skin fibroblast cell cultures [11,15-16]. Meanwhile, to test the level of safety against the human body, it is carried out *in vivo*. The *in vitro* assay is fast, reliable, and not harmful [13]. The parameter representing the quality of sunscreens is the sun protection factor (SPF), which can be determined using the UV-Vis spectrometer. The SPF value is the ratio the minimal dose of erythema for skin protected by sunscreen over the minimal dose of erythema for non-sunscreen-protected skin [10,13].

The *in vitro* SPF value was determined using Eq. (1), a method used by Ngurah et al. [15]. The absorbance of the sample was determined at 5 nm intervals in the length range of 290–320 nm.

$$\text{SPF spectrophotometric} = A^{\text{Avg}-\text{Abs}} \quad (1)$$

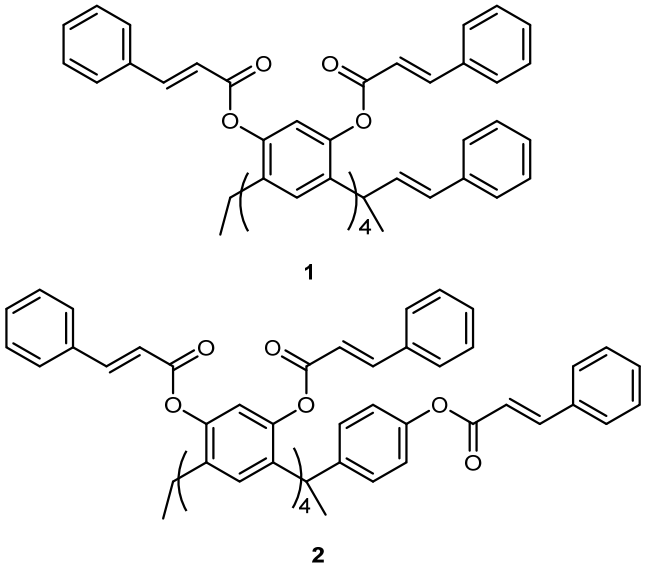

Fig 1. Potential sunscreens based on calix[4]resorcinarene derivatives

The preliminary structural analysis using Hyperchem shows that the compounds that have the potential as sunscreens are calix[4]resorcinarene derivatives namely C-styrylcalix[4]resorcinaryl octacinnamate **1** and C-phenylcalix[4]resorcinaryl dodecacinnamate **2** (Fig. 1). Compounds **1** and **2** have many conjugated double bonds with carbonyl group (C=O). The more conjugated bonds, the higher the ability to absorb UV-B radiation [17-18]. Based on the previous explanation, we report herein the synthesis, sunscreen and toxicity assays towards calix[4]resorcinarene derivatives **1** and **2**.

■ EXPERIMENTAL SECTION

Materials

Materials used were ethanol, dimethyl sulfoxide, cinnamoyl chloride, pyridine, parasol, chloroform, 3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide, RPMI 1640 and Vero-kidney cells. The precursors of C-styrylcalix[4]resorcinarene and C-(4-hydroxy)phenylcalix[4]resorcinarene were synthesized by Ngurah et al. [19].

Instrumentation

Instruments used were melting point apparatus WRS 200, ^{1}H-NMR spectrometer (Agilent 400), LC-MS spectrometer (TSQ Vantage), infrared spectrophotometer (Shimadzu, Prestige 21), and UV-Vis spectrophotometer (Genesys 150).

Procedure

Synthesis of C-styrylcalix[4]resorcinaryl octacinnamate 1

C-Styrylcalix[4]resorcinarene **3** (4 g, 4.5 mmol) and cinnamoyl chloride **4** (8.32 g, 50 mmol) were mixed in 25.0 mL of pyridine in an ice bath. After heating the mixture at 65 °C for 3 h, the mixture was cooled, and the reaction was quenched by adding 150 mL of water. The formed precipitate was filtered and dried at 50 °C in an oven. The melting point of the reaction products was determined using a melting point apparatus WRS 200. The product was elucidated by infrared, proton nuclear magnetic resonance, and Liquid Chromatography-Mass spectrometers.

Synthesis of C-phenylcalix[4]resorcinaryl dodecacinnamate 2

C-Phenylcalix[4]resorcinaryl dodecacinnamate **2** was prepared from the starting materials of C-(4-hydroxy)phenylcalix[4]resorcinarene **5** (4 g, 4.7 mmol) and cinnamoyl chloride **4** (12 g, 72 mmol) using the similar procedure for the synthesis of **1**.

Sunscreen test

Solution of calix[4]resorcinarenes **1** and **2** was prepared at various concentrations of 5, 10, 15, 20, and 25 ppm in chloroform. The absorbance of each solution was determined using UV-Vis spectrophotometer. The absorbance value (A) was plotted with the wavelength to obtain the maximum wavelength (λ_{max}). The absorbance of samples **1** and **2** was determined in the wavelength of 280–400 nm and chloroform was used as blank. The SPF value was calculated using Eq. (2):

$$\text{Log SPF} = \text{Average of absorbance, SPF} = 10^{A} \quad (2)$$

Cytotoxicity test

The cytotoxicity assay was carried using MTT assay [20]. Calix[4]resorcinarenes **1** and **2** were tested for their cytotoxicity against renal Vero cells. The reagent used was 3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide while the cell concentration used was 104 cells/mL in RPMI 1640 medium. Samples **1** and **2** with various concentrations of 1000, 500, 250, 125, 31.25 and 15.125 g/mL were then added. Parasol sunscreen was used as the positive control. In MTT assay, cells that do not die due to the exposure to ultraviolet light will react with 3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide salt to form a purple color. The ones that are still alive are expressed as optical density (OD). The more cells that are alive, the higher the OD value. The measurements were carried out at a wavelength of 595 nm in triplicate.

■ RESULTS AND DISCUSSION

Synthesis of C-styrylcalix[4]resorcinaryl Octacinnamate 1

C-Styrylcalix[4]resorcinarene octacinnamate **1** was synthesized from C-styrylcalix[4]resorcinarene **3** via esterification reaction using pyridine as a catalyst and solvent (Fig. 2). The product was obtained as a light brown solid in 68% yield with the melting point of 247 °C. The product was further characterized using an IR, ^{1}H-NMR and MS spectrometers.

The infrared spectrum of **1** is displayed in Fig. 3. The absorption at 1728 cm^{-1} indicates the presence of the carbonyl ester group suggesting that the formation of **1** has occurred.

The ^{1}H-NMR spectrum (in DMSO-d_6) shows six types of protons (Fig. 4). The shift at 5.7 ppm is due to the methylene bridge proton, while the peaks at 6.6 and 6.9 ppm represent the methylene proton of styryl group attached to the methylene bridge. The peaks at 7.7–7.5 ppm belong to the aromatic protons from calix[4]resorcinarene moiety and cinnamoyl group.

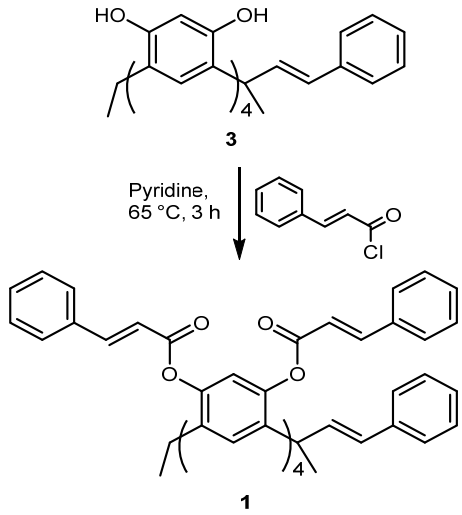

Fig 2. Synthesis of calix[4]resorcinarene **1**

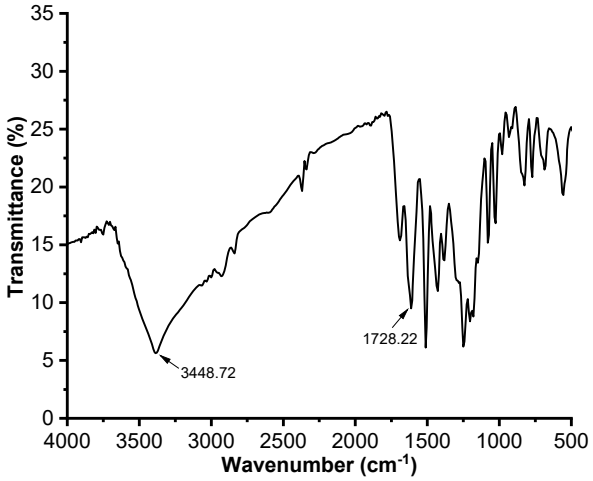

Fig 3. IR spectrum of calix[4]resorcinarene **1**

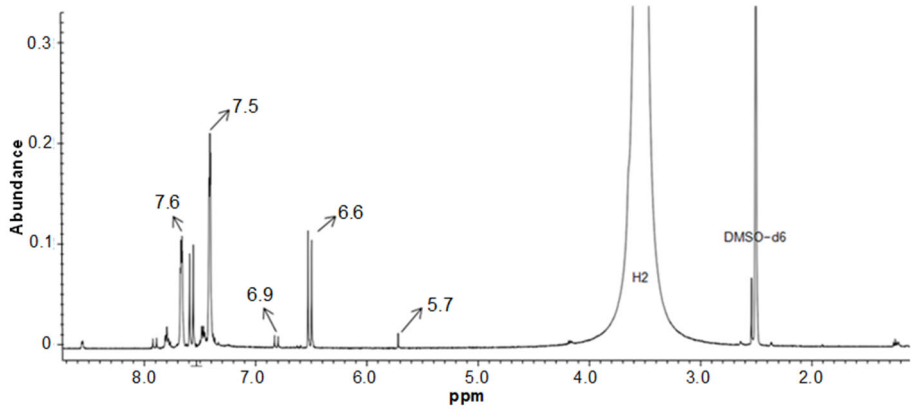

Fig 4. [1]H-NMR spectrum of calix[4]resorcinarene **1**

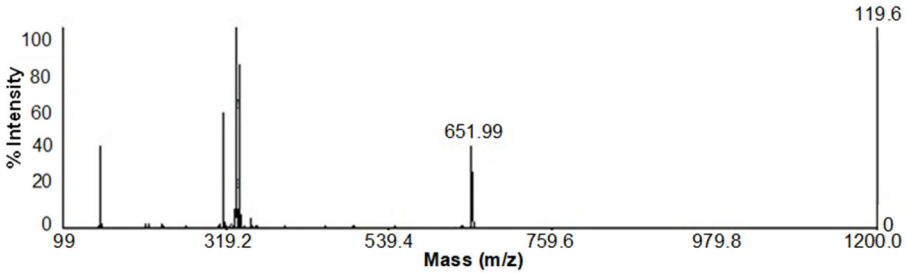

Fig 5. Mass spectrum of calix[4]resorcinarene **1**

The molecular weight of **1** was determined using LC-MS spectrometer. The mass spectrum of **1** shows that the molecular mass of the target compound does not appear. Still, the appearance of the base peak (*m/z* 651) indicates the release of 4 cinnamic and four cinnamoyl groups (Fig. 5). Based on the melting point analysis and characterization using the IR, [1]H-NMR and LC-MS spectrometers, it can be indicated that calix[4]resorcinarene **1** has been produced.

Synthesis of C-Phenylcalix[4]resorcinaryl Dodecacinnamate 2

C-Phenylcalix[4]resorcinaryl dodecacinnamate **2** was synthesized via esterification of C-(4-hydroxy) phenylcalix[4]resorcinarene **5** and cinnamoyl chloride **4** using pyridine as a catalyst and solvent (Fig. 6). The product was obtained as light-yellow solid in 69% yield with melting point of 268 °C. The product was further characterized using IR, [1]H-NMR and MS spectrometers.

The IR spectrum of **2** shows strong absorption at 1689 cm^{-1} represents the ester carbonyl group indicating that the esterification has taken place (Fig. 7). The peak at 3387 cm^{-1} is caused by impurities.

The [1]H-NMR spectrum (Fig. 8) shows six types of protons, where the methylene protons from methylene bridge and cinnamoyl group appear at 6.4 and 7.4 ppm, respectively. The peaks at 7.6 and 7.7 ppm represents the

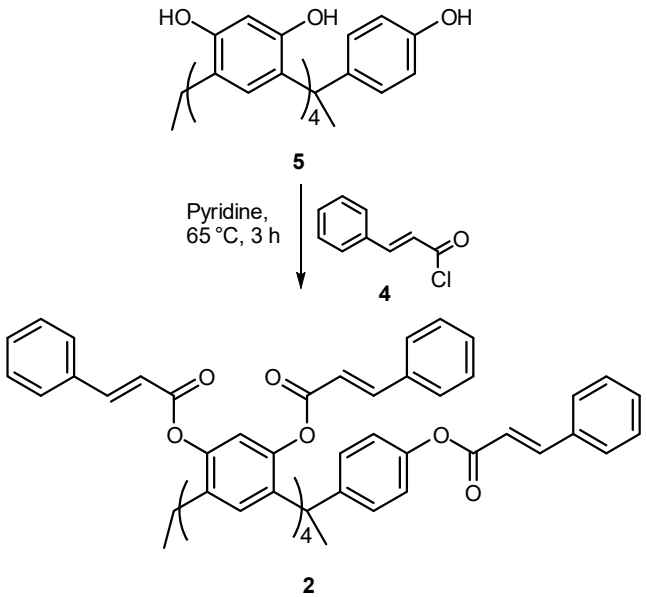

Fig 6. Synthesis of calix[4]resorcinarene **2**

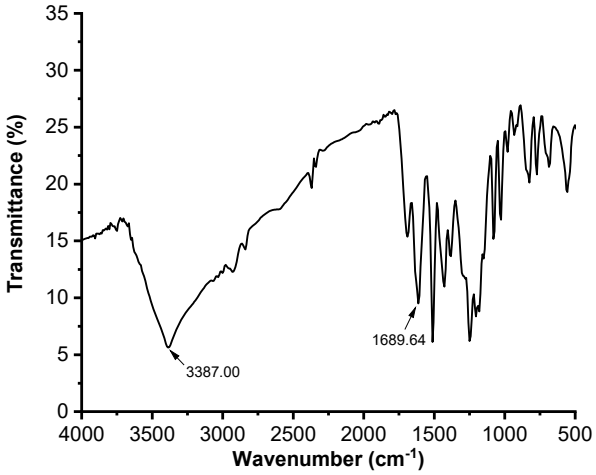

Fig 7. IR spectrum of calix[4]resorcinarene **2**

aromatic protons of calix[4]resorcinarene scaffold and cinnamoyl group, respectively.

LC-MS spectrometer was employed to determine the molecular weight of product **2** (Fig. 9). The mass spectrum of **2** shows that the molecular mass of the target compound does not appear, but the appearance of peak at m/z of 652.10 indicates the release of 10 cinnamoyl groups. Based on the melting point analysis and characterization using the IR, ^{1}H-NMR and LC-MS spectrometers, it can be indicated that calix[4]resorcinarene **2** has been produced.

In Vitro Sunscreen Activity Test

The sunscreen activity test of calix[4]resorcinarenes **1** and **2** by UV-Vis spectrophotometer shows that the higher the concentration, the higher the average absorbance value (A^{Avg}) in the wavelength range of 290–320 nm. The higher the average absorbance indicated that compounds **1** and **2** are more active to absorb UV-B, giving calix[4]resorcinarenes **1** and **2** higher SPF value (Fig. 9).

The types of protection represent the number of UV-B rays absorb by the sunscreens. They can be classified into minimal (less than 50% of absorbed UV-B ray), moderate (60%), maximum (96%) and ultra (99%) protections. Fig. 10 shows that the types of protection at 5, 10 and 15 ppm are minimal, moderate and maximum, respectively. In addition, the ultra-protection is observed

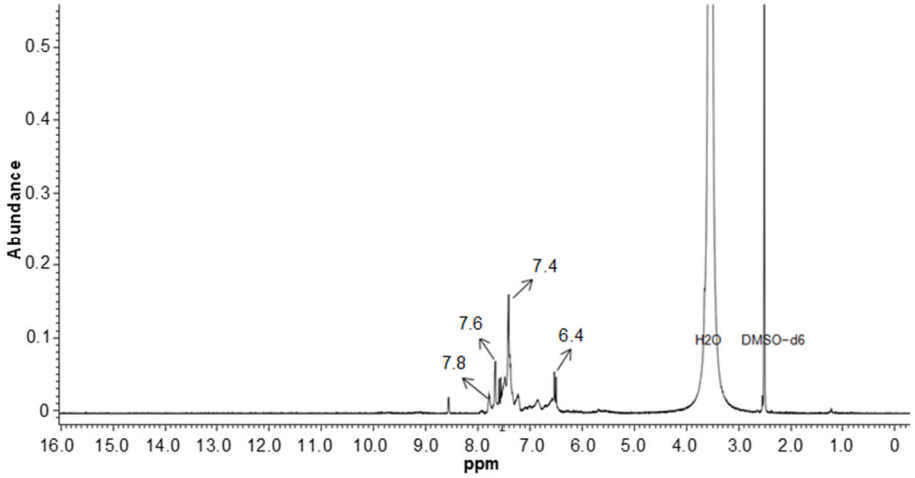

Fig 8. ^{1}H-NMR spectrum of calix[4]resorcinarene **2**

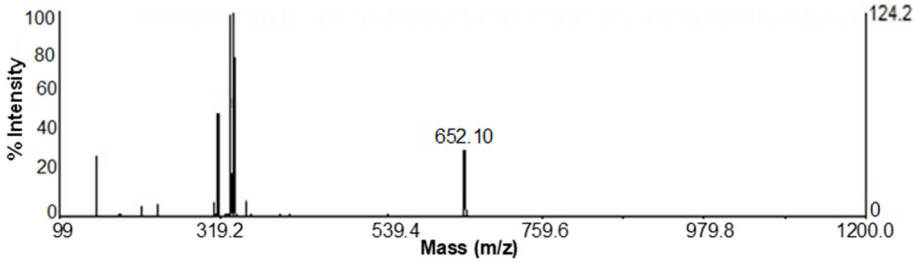

Fig 9. Mass spectrum of calix[4]resorcinarene **2**

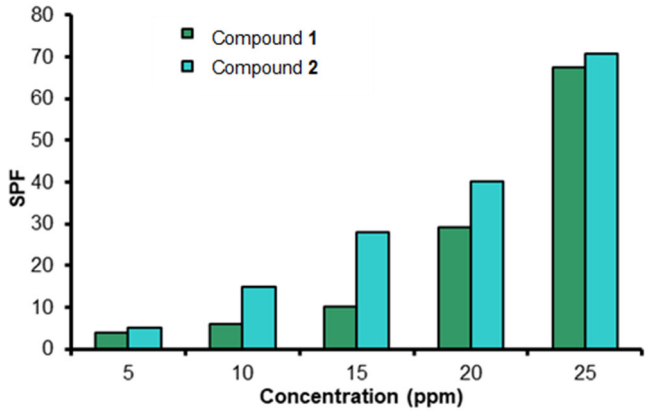

Fig 10. SPF values of calix[4]resorcinarenes **1** and **2** at various concentration

at concentration of 20 and 25 ppm. The results shows that the SPF value of **2** (70.58) is higher than that of **1** (67.45) since compound **2** bears more cinnamoyl groups, which show strong absorption in the UV-B region.

Cytotoxicity Test

Calix[4]resorcinarenes **1** and **2** as well as parasol (positive control) were tested for cytotoxicity against the kidney Vero cells (Table 1). The results show that the higher dosage of samples gives higher average absorbance of sample. As the results, the samples are more viable against Vero cells. The living cells are expressed as OD. The more cells that are still alive leads the higher OD value.

The amount of living cells is calculated by Eq. (3):

$$\%\ \text{Living cells} = \frac{(C-B)}{(A-B)} \times 100\% \qquad (3)$$

where, A = cell control absorbance, B = media control absorbance, C = sample absorbance

The living cell percentage data of samples **1**, **2** and parasol are then processed using Excel program to obtain the IC_{50} value. Fig. 11 shows that the higher the concentration of **1** and **2**, the higher the percentage of dead Vero cells (the compounds are more active against Vero cells). At 15-500 ppm, the percentage of dead Vero cells are almost the same for both **1** and positive control. At 1000 ppm, the positive control is, however, toxic to Vero cells, while **1** shows better toxicity profile since **1** is already adaptive to Vero cells. Compound **2** leads more dead cells than **1** and parasol at all concentrations. The cinnamate ester groups of **1** and **2** may undergo hydrolysis to give free phenolic groups, which are toxic to kidney cells. After the hydrolysis reaction, compound **2** bears more phenolic groups than compound **1**. The cytotoxicity test shows that the IC_{50} value of **1** (1468.2 µ/mL) is higher than that of **2** (676,1 µ/mL) and parasol control (758.7 µ/mL). The results shows that **1** has lower toxicity than parasol and can be used as a sunscreen ingredient.

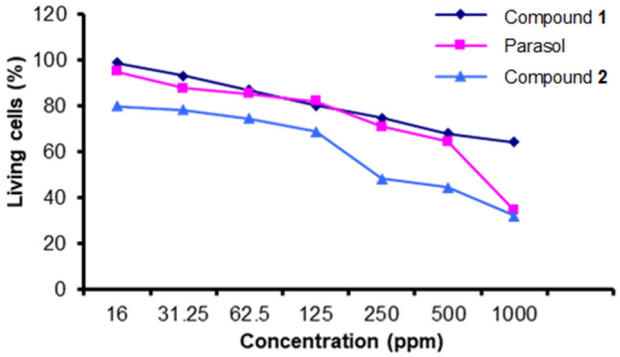

Fig 11. Percentage of viable Vero cells at various concentrations

Table 1. Average absorbance of samples **1**, **2**, positive control, cell control and media control

Dosage (µg/mL)	Average absorbance				
	1	**2**	Parasol	Cell control	Media control
1000	0.336	0.206	0.216	0.48	0.076
500	0.351	0.256	0.337		
250	0.378	0.271	0.363		
125	0.4	0.354	0.408		
62.5	0.427	0.378	0.421		
31.25	0.453	0.393	0.431		
15.625	0.476	0.399	0.46		

■ CONCLUSION

To conclude, two calix[4]resorcinarene derivatives **1** and **2** have been synthesized. These compounds can absorb UV-B ray as depicted by the high SPF value at concentration of 5 to 25 ppm. Calix[4]resorcinarene **1** is not toxic to kidney Vero cells so it can be used as the sunscreen ingredient. Calix[4]resorcinarene **2** also has the potential to be developed as the sunscreen product, although it is slightly more toxic than positive control of parasol.

■ ACKNOWLEDGMENTS

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