http://dx.doi.org/10.4314/ajtcam.v12i2.19

IN VITRO WOUND HEALING ACTIVITY OF *SAPIUM INDICUM* WILLD LEAF EXTRACTS

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Abstract

Background: *Sapium indicum* Willd (Euphorbiaceae) is traditionally used to accelerate wound healing process in Malay community.
Objective: To evaluate wound healing potential of aqueous (AESI) and ethanolic extract of *S. indicum* leaves (EESI) using cell migration model.
Methods: AESI and EESI were prepared using maceration techniques. The extracts were subjected to phytochemical screening, cytotoxicity (MTT assay) and scratch assay.
Results: AESI contained saponins and tannins while EESI contained triterpenes, tannins and proteins. Based on MTT assay, AESI and EESI exhibited **IC_{50} = 16.3 and 70.9 µg/mL, respectively**. In the scratch assay, EESI caused significant ($P< 0.05$) concentration-dependent migration on 3T3 L1 cells whereas AESI exerted concentration-independent effect.
Conclusion: The leaves of *S. indicum* exhibited wound healing potential and contained phytochemicals that may contribute to the activity. These findings would support potential use of *S. indicum* as wound healing plant.

Keywords: *Sapium indicum*, cell migration, *in vitro* wound healing

Introduction

Wound healing is a complex process that is immediately initiated in response to an injury in order to achieve final repair. The wound healing process is a stepwise process that involves a series of biochemical and cellular reactions, which consists of different phases such as hemostasis, inflammation, proliferation and remodeling or maturation (Murti et al., 2012). Normal wound healing progresses in a predictable and timely manner. Whereas in abnormal progression, healing may progress inappropriately resulting in either a chronic wound or pathological scarring (Thakur et al., 2011). Nowadays, wound healing is still considered as one of the major concerns among health care practitioners and scientists. Poor wound healing not only cause trauma to the patient but also increases the burden of financial resources required for cost-effective management within the health care system (Bowler et al., 2001).

This research aims at discovering new and natural wound healing agents from medicinal plants. The plants currently being tested for its wound healing claim is *Sapium indicum* Willd. (Euphorbiaceae) locally known to the Malays as "Gurah". Traditionally, the tender leaves of *S. indicum* are squeezed with some limestone paste to form a froth that is applied directly to the wound. The use of limestone paste that time is believed to neutralize the latex come from their leaves.

Other traditional uses of *S. indicum* include the treatment of fever, gonorrhea and toothache (Burkill, 1935; Purwaningsih, 1991). The decoction prepared from *S. indicum* is useful as a purgative and emetic, and also as medication for insanity and hydrophobia (Burkill, 1935; Gautam et al., 2007). Despite traditional claims, the medicinal uses of *S. indicum* are sometimes overshadowed by the fact that its latex is poisonous and can cause blisters. Moreover, the fruits are commercially used as fish poison due to presence of aesculetin in the fruits (Gautam et al., 2007). In an attempt to contribute to the establishment of the medicinal potential of *S. indicum*, we studied the wound healing activity of the aqueous and ethanolic extracts of the leaves of *S. indicum* using *in vitro* scratch assay.

Materials and methods

Chemicals and Reagents

Dulbecco's Modified Eagle Medium (Sigma, USA), fetal bovine serum (JR Scientific Co., US), penicillin streptomycin (Gibco, USA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT) (Calbiochem, Germany), and platelet derived growth factor-BB (Calbiochem, Germany) were used in the study. All other chemicals and solvents used in this study were of reagent or analytical grade.

Plant Material

Fresh leaves of *S. indicum* were collected in Kampung Al or Melintang, Al or Setar, Kedah, Malaysia around October, 2012 and had been identified by Dr. Shamsul Khamis of University Putra Malaysia. The voucher specimen (MT13-01) was deposited in the herbarium at Kulliyyah of Pharmacy, International Islamic University Malaysia, Kuantan, Pahang, Malaysia (Figure 1).

Water extraction

The finely chopped fresh leaves (100 g) were soaked in distilled water (1:4; w/v) and placed in an incubator shaker for 8 hr before filtering with gauze. The supernatant was collected and centrifuged at 27°C for 10 min at 6000 rpm. The centrifuged supernatant was then

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freeze-dried to obtain the final extraction in powder form. The weight of the final form of the extract was recorded to determine the yield. The powder was kept in a falcon tube at -80°C until further analysis.

Ethanol extraction

The finely chopped fresh leaves (100 g) were soaked in absolute ethanol (1:3; w/v) for three days on a shaker. After three days, the supernatant was collected and the residue was treated with the same extraction procedure twice. The collected supernatants were pooled together and the solvent was evaporated using a rotary evaporator. The weight of the final dried crude extract was recorded to determine the yield. The extract was kept in a falcon tube at -80°C before further analysis.

Phytochemicals Screening of the Extracts

Different groups of phytochemical constituents such as alkaloids, flavonoids, saponins, triterpenes, steroids, tannins and proteins were detected qualitatively in both extracts using standard phytochemical screening procedures (Brain et al., 1975, Shaik, 2011, Shadid-Ud-Daulaet al., 2009).

Dragendorff test for alkaloid detection

A few drops of concentrated hydrochloric acid (HCl) were added to 2 mL of each sample solution in the test tubes. Then, Dragendorff reagent was added to the samples and the formation of orange precipitate indicates the presence of alkaloid.

Shinoda test for flavonoid detection

A few pieces of magnesium coils were dropped into 2 mL of each sample solution in the test tubes and 0.5 mL of concentrated hydrochloric acid (HCl) was then added to the mixture. The appearance of orange to red color indicates positive result of the flavonoid presence in the sample.

Frothing test for saponin detection

2 mL of the each sample solution was added with 2 mL distilled water in a test tube and the mixture was vigorously shaken. The formation of froth indicates the positive result for saponin content. The result is indicated as weakly positive if froth less than 1 cm high, positive if froth 1.2 cm high, and strongly positive if the froth is greater than 2 cm.

Libermann-Burchard test for steroid and terpenoid detection

3 to 5 drops of acetic anhydride was added to the 2 mL of each sample solution and mixed properly. 1 to 2 drops of concentrated sulphuric acid was mixed slowly from the wall of the test tube. Blue and purple colorations indicate the presence of terpenoid and steroid respectively.

Iron (III) Chloride ($FeCl_3$) test for tannin detection

2 mL of each sample solution was added with a few drops of $FeCl_3$ in the test tubes. Brownish green to blue black coloration indicates the positive result of tannin.

Biuret test for protein detection

The extract solutions were treated with 1 mL of 10% sodium hydroxide solution and heated. A drop of 0.7% copper sulphate solution was then added. Formation of purplish violet color indicates the presence of protein.

Cell Culture and Cytotoxicity

Frozen 3T3-L1 cell line (ATCC-CL-173) which was obtained from the American Type Culture Collection (ATCC) was quickly thawed in a 37ºC water bath. The cells were transferred to a T-flask containing 9 mL of complete media [Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS (Fetal Bovine Serum) and 2% penicillin and streptomycin], and then incubated at 37ºC in a 5% CO_2 humidified incubator. The media was changed one day after plating and every two to three days thereafter. The cells were propagated until the density reached 70-80% confluence before subculture (Canfield, 2011).

To check cytotoxicity, 100 µL of 3T3 L1 cells suspension were seeded into a 96-wells cell plate and incubated (24 hr). At the end of the incubation period, the medium was replaced by 100 µL of supplemented DMEM in the presence or absence of different concentrations of AESI or EESI and the plate was incubated for another 24 hr. Then, 20 µL of 5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT) solution was added to each well, then further incubated for another 4 h at 37ºC (Steenkamp et al., 2004). Finally, 100 µL of dimethyl sulfoxide (DMSO) was added to each well and the plate was gently shaken for 10 min to dissolve the purple formazan crystals formed. Cell growth was evaluated using a micro-plate reader at 570 nm with reference wavelength of 630 nm. The absorbance readings of the test determine the suitable dosage to be used in the scratch assay test. The test was performed in triplicate.

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Scratch Assay Test

spreading and migration capabilities of 3T3 L1 cells were assessed using scratch wound assay, which measures the expansion of a cell population on surfaces as described by Fronza et al. (2009). The cells were seeded into 6-well tissue culture dishes in DMEM containing 10% FBS (Fetal Bovine Serum) and 2% penicillin and streptomycin. After the cell shave nearly formed a confluent cell monolayer, a linear wound was generated in the monolayer using a sterile 100 µL plastic pipette tip. Any cellular debris was removed by washing the wells with phosphate buffer saline (PBS). The medium (DMEM) used consisted of either dimethyl sulfoxide (<0.01% used as solvent control), platelet derived growth factor (PGDF; 0.002 µg/mL) (as positive control), or the crude extracts (0.19 µg/mL, 1.56 µg/mL, and 12.5 µg/mL). The cells were then incubated for 16 hr at 37°C with 5% CO_2. The scratched cell layers incubated under the different conditions were then photographed to estimate relative cell migration. The data were analyzed using CapturePro Version 2.5 for Progres® microscope camera from Jenoptik Laser, Optik, Systeme GmbH. The experiment was performed in triplicate.

Statistical Analysis

Statistical evaluation was carried out with IBM SPSS statistics version 20 and with Microsoft Office Excel 2007. The data was expressed as mean ± S.D. Significant differences between the treated groups and the control were determined by one-way ANOVA using Kruskal-Wallis test, at a significance level of $P< 0.05$.

Results

Extraction and Phytochemical Screening

The freeze-dried aqueous extract of *S. indicum* (AESI) appears as a light brown powder while the ethanolic extract of *S. indicum* (EESI) was sticky and dark color. The yield of AESI (10.2 %) and EESI (15.7 5) were calculated based on the amount of yield obtained form the extraction. AESI tested positive result for the presence of saponins and tannins while EESI demonstrated the presence of terpenoids, tannins and proteins (Table 1).

Cytotoxicity Effect *S. indicum* Extracts

Based on the cytotoxicity test using different concentrations of AESI and EESI, IC_{50} falls within the range of 12.5-25 µg/mL (IC_{50} = 16.3 µg/mL) and 50-100 µg/mL (IC_{50} = 70.9 µg/mL), respectively (Figure 2 and 3). Based on the cell viability study, three concentrations (0.19, 1.56 and 12.5 µg/mL) were chosen for the scratch assay investigation. The reason for choosing 12.5 µg/mL of extract as the highest concentration for the scratch assay was because at this concentration, the percentage of cell viability still exceeded the 50% level when pre-treated with AESI, but decreased drastically to below 50% when the concentrations were increased from 25 to 100 µg/mL. Although the cells treated with EESI exerted viability exceeding the 50% level until the concentration of 50 µg/mL, the highest concentration 12.5 µg/mL was chosen for the scratch assay to enable comparison between AESI and EESI.

Wound Healing Effect of *S. indicum* Extracts

Using the scratch assay, cell migration in response to an artificial injury was observed under phase-contrast microscope (Figure 4) and the rate of cell migration was calculated.The results on effects of AESI and EESI using the scratch assay are shown in Table 2. Pre-treatment with both extracts caused significant ($P < 0.05$) changes in the relative cell migration compared to the control group (treated with 0.01% DMSO). However, only EESI exerted cell migration effect at all concentrations tested, while AESI at 1.56 µg/ml concentration of extract did not significantly affect the cell migration. EESI said to have concentration-dependent migration of 3T3 L1 cells since relative cell migration of the extract increase proportionally with concentration increment, while AESI showed the opposite result. The positive control group receiving 0.002 µg/ml of PDGF also exerted significant ($P< 0.05$) cell migration effect.

Figure 1: The image of voucher specimen of *Sapium indicum*

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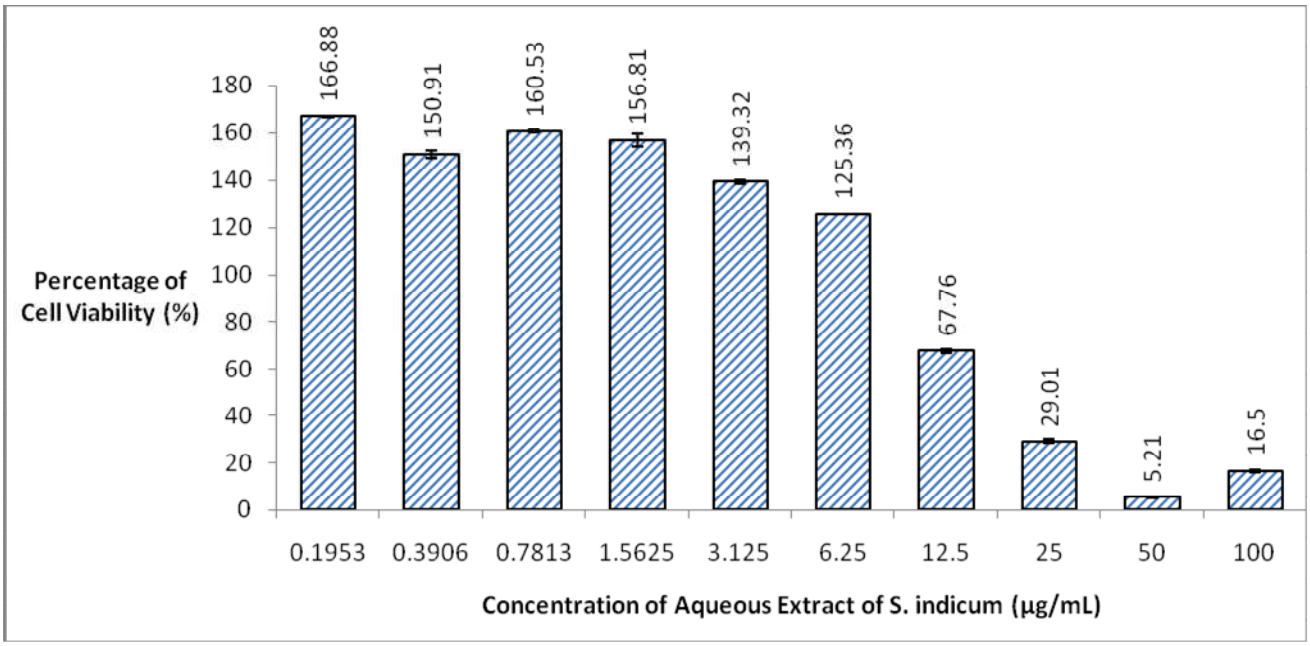

Figure 2: Cytotoxicity effect of AESI on 3T3-L1 adipocytes

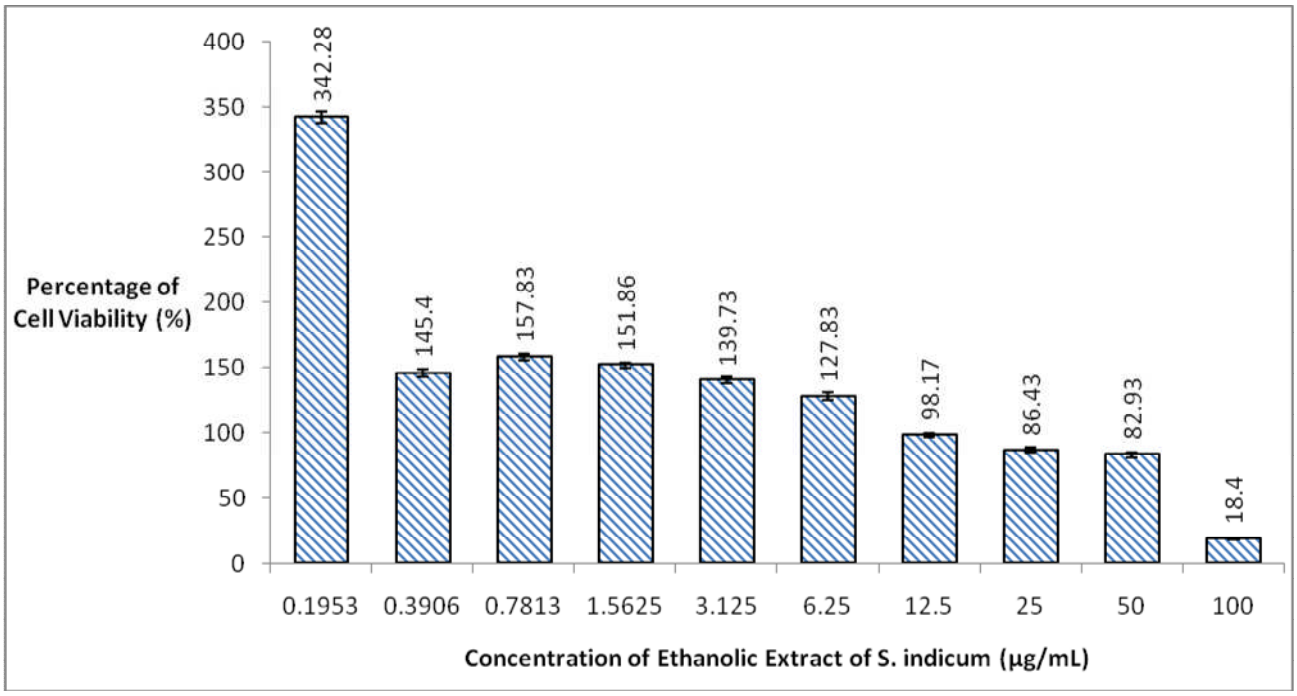

Figure 3: Cytotoxicity effect of EESI on 3T3-L1 adipocytes

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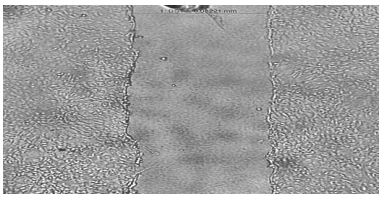
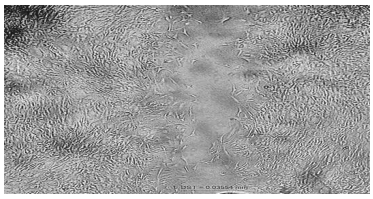
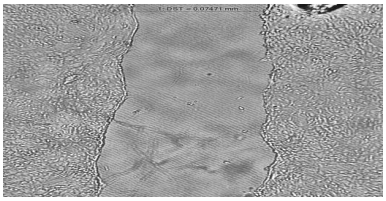
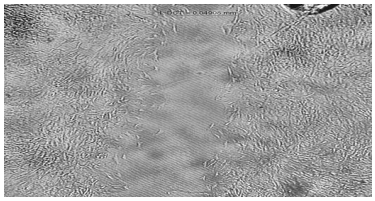
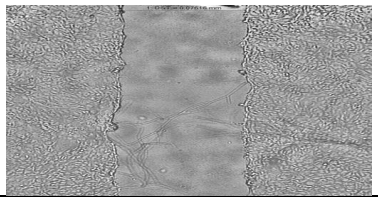
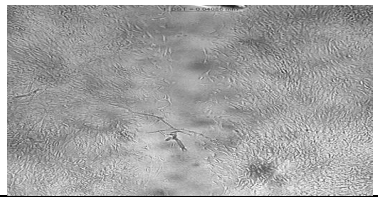
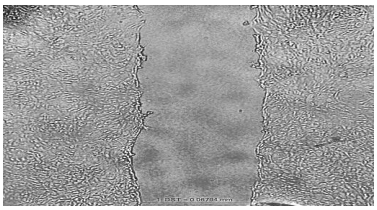
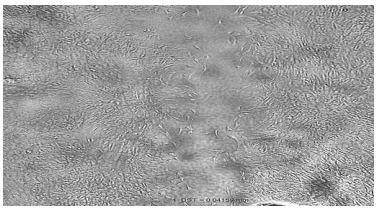
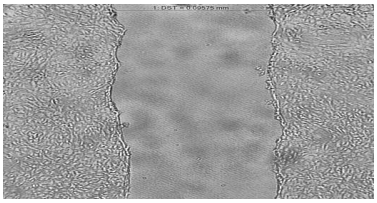
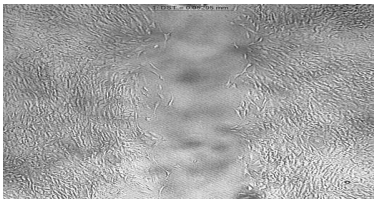
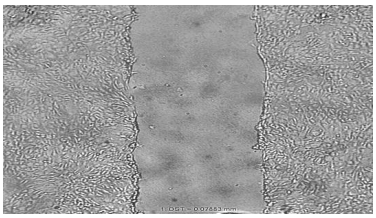
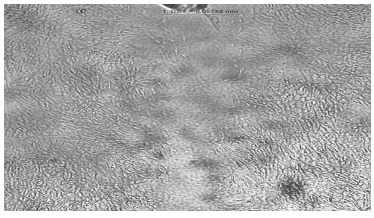
Treatment group	Concentration (µg/ml)	Observation	
		0 hour after wounding	16 hours after wounding
0.01% DMSO (solvent control)	-		
PDGF (Positive control)	0.002		
AESI	0.19		
	12.5		
EESI	0.19		
	12.5		

Figure 4: The phase-contrast microscope images of 3T3 L1 fibroblasts pre-treated with AESI and EESI followed by the potential *in vitro* wound healing evaluation using the scratch assay. Measurements were made at two interval, namely 0 and 16 h after the induction of wound. Effect of AESI and EESI, at 1.56 µg/ml, was not shown.

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Table 1: Phytochemical constituents of AESI and EESI.

Phytochemical	AESI		EESI	
	Observation	Results	Observation	Results
Alkaloids	No yellow precipitation	-	No yellow precipitation	-
Flavonoids	No color changes	-	No color changes	-
Saponins	Formation of froth	+	No froth formation	-
Steroids	No color changes	-	No color changes	-
Terpenoids	No color changes	-	Bluish color formation	+
Tannins	Formation of blue black coloration	+	Formation of blue black coloration	+
Proteins	No color changes	-	Violet color formation	+

Note. Minus sign (-) refers to the absence of the respective biochemical compound while plus sign (+) refers to the presence of the respective biochemical compound.

Table 2: *In vitro* wound healing effect of AESI and EESI measured as relative cell migration (mm) using the scratch assay

Treatment Group	Concentration (µg/mL)	Relative Cell Migration (mm)					Mean±S.D. (mm)
		1	2	3	4	5	
0.01% DMSO	-	0.021	0.011	0.025	0.025	0.024	0.021 ± 0.00533
PDGF	0.002	0.026	0.011	0.033	0.019	0.030	0.033 ± 0.00386*
AESI	0.19	0.031	0.043	0.036	0.035	0.036	0.036 ± 0.00378*
	1.56	0.023	0.025	0.031	0.039	0.030	0.030 ± 0.00531
	12.5	0.031	0.032	0.041	0.044	0.026	0.035 ± 0.00681*
EESI	0.19	0.033	0.043	0.031	0.039	0.041	0.037 ± 0.00480*
	1.56	0.044	0.037	0.048	0.030	0.031	0.038 ± 0.00705*
	12.5	0.043	0.043	0.043	0.040	0.042	0.042 ± 0.00134*

* Data differed significantly ($P < 0.05$) when compared to the 0.01% DMSO-treated group

Discussion

It has been reported that 80% of the population in some Asian and African countries depend on traditional medicine for their primary health care (World Health Organization, 2008). However, some traditional medicines, particularly plant based, have not been scientifically proven

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to be effective nor safe for consumption. Hence, research into traditional medicine should be continued in order to provide knowledge on the safety and effectiveness of such plant based treatments.

In the present study the effect of *S. indicum* leaves for the treatment of wound healing was determined using *in vitro* model. It was found that AESI was more cytotoxic than EESI with IC_{50} range between 12.5 to 25 µg/mL and between 50 to 100 µg/mL, respectively. From this assay, three concentrations of AESI and EESI (0.19, 1.56 and 12.5 µg/mL) were selected and tested for its wound healing potential using in vitro scratch assay. It was found that EESI exerted concentration-dependent wound healing activity at all doses tested while the AESI exerted concentration-independent activity.

In folklore medicine, medicinal plants have been used extensively to facilitate wound healing. The wound healing properties performed their action due to their phyto-constituents. Phyto-constituents such as tannins, saponins, terpenes and triterpenes are some of the well-studied group of compounds that assist the wound healing process in many ways. Preliminary screening for phyto-constituents in the respective extracts revealed the presence of saponins and tannins in AESI and triterpenes, tannins and proteins in EESI. These findings correlate with the above notation and support the report by Hiwot (2010) on the ability of such mention phyto-constituents in healing of the wounds. The high potential of medicinal plants in assisting wound healing has inspired much research aimed at validating the claims and discovering mechanisms to explain the potential of these herbs in wound repair processes.

Conclusions

The study suggested that *S. indium* leaves exhibited wound healing activity *in vitro.* The ethanol extract improved wound healing activity might be due to the presence of several phyto-consituents such as saponins, tannins and triterpenes. Further studies are recommended to determine the potential wound healing activity of *S. indicum* leaves in animal model and to identify the responsible bioactive compounds.

Acknowledgement

The authors are thankful to the International Islamic University Malaysia, Malaysia for providing financial support and the facility to carry out this research.

Declaration of Conflict of Interest: The authors declare no conflict of interest.

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