USE OF *ADENIA CORDIFOLIA* EXTRACT FOR WOUND HEALING

FIELD OF INVENTION

This invention generally relates to plant extracts. More particularly, the invention relates to the use of a plant extract derived from *Adenia cordifolia* (*A. cordifolia*) for wound healing.

DESCRIPTION OF THE PRIOR ART

A wound is an injury to the body that typically involves laceration or breaking of the epidermis, due to external violence, accident or surgery. When a wound is inflicted, the body will respond to the wound naturally by initiating a wound healing process which consists of several distinct, yet overlapping phases: inflammatory phase, proliferative phase and tissue remodeling phase.

Proper wound care and treatment is necessary to prevent infection and other complications, and also to speed up the healing process.

Current standard-of-care in the treatment of wounds comprises solely of prescribing a variety of synthetic medications, none of which can be said to have been singularly effective in healing wounds.

Corticosteroids are artificial versions of hormones normally produced by the adrenal glands and are often used as part of wound treatment to provide relief for inflammation. Consumption of high dosage or prolonged use of corticosteroids carry a risk of side effects such as increased risk of infections. There are clinical reports showing that administration of corticosteroids has no important effect on wound healing, and in fact, corticosteroids inhibit the wound healing process.

Non-steriodal anti-inflammatory drug (NSAID) is another common medication prescribed in the treatment of wounds to reduce inflammation, pain, fever, stiffness and swelling. However, NSAIDs have known anti-proliferative effect on blood vessels

and skin that actually delays wound healing. For example, systemic use of ibuprofen has demonstrated an anti-proliferative effect on wound healing, resulting in decreased number of fibroblasts, weakened breaking strength, reduced wound contraction, delayed epithelialization and impaired angiogenesis. NSAIDs also have anti-clotting properties. For example, COX-2 inhibitors interfere with platelet function, which leads to diminished cell function in the clotting process during the inflammatory phase of wound healing.

Antioxidants are important in wound healing because it is a molecule that inhibits oxidation of other molecules. Vitamin C is a potent water-soluble antioxidant source. However, consumption of high amounts of vitamin C can lead to adverse effects, for example, constipation, diarrhea or hypersensitivity reactions.

Another alternative is the topical medication, liposome-encapsulated buflomedil hydrochloride, to quicken neovascularisation in wound healing and accelerate wound closure. However, the delivery of this drug faces biological challenges as liposomes encounter several of the human body's defense systems intended at recognition, neutralization and elimination of invading substances. When this occurs, the optimal liposome function is disrupted, and drug delivery becomes ineffective.

Another treatment currently available for wound healing is cell-based therapy where cellular material is injected into a patient. Adult primary cells are developed *in vitro* and then implanted directly into the patient. The primary cells are able to multiply and differentiate into specific type of cells that are essential in healing of wounds. However, this treatment is associated with a number of limitations such as risks of immune rejection and infection transmission.

As seen above, all the available medical options typically only address one or two phases of wound healing. In light of the shortcomings and various side effects of existing medications for the treatment of wounds, there is a need to develop a medication that singularly and effectively addresses all phases of wound healing with minimal side effects.

This invention thus aims to alleviate some or all of the problems of the prior art.

SUMMARY OF INVENTION

In an aspect of the invention, there is provided a plant extract derived from the stem of *Adenia cordifolia*. The extract comprises (E)-9-octadecenoic acid ethyl ester ($C_{20}H_{38}O_2$) and 9,12-octadecadienoic acid ethyl ester ($C_{20}H_{36}O_2$).

In another aspect of the invention, there is provided use of a plant extract derived from the stem of *Adenia cordifolia* in the manufacture of a medicament for administration to a human for wound healing.

In an embodiment, the medicament may be administered orally in the form of pellets, tablets, capsules, powders, granules, suspensions or emulsions.

In another embodiment, the medicament may be administered topically in the form of lotions, ointments, creams, powders, gels or transdermal patches.

In yet another embodiment, the medicament may be administered both orally and topically, concurrently.

According to an embodiment, the plant extract may be an ethanol extract and may be an extract according to claim 1.

In another aspect of the invention, there is provided a medicinal wound healing composition comprising a plant extract derived from the stem of *Adenia cordifolia*.

The extract may be an extract according to claim 1.

The present invention provides a plant extract for healing wounds in humans that has increased efficacies relative to standard-of-care treatment, without the undesirable side effects. The plant extract has substantial multifactorial potency for promoting wound healing because of its anti-oxidant and pro-angiogenic properties.

Various other advantages of the plant extract of this invention will be further elaborated in the following pages.

BRIEF DESCRIPTION FOR THE DRAWINGS

The invention is illustrated, although not limited, by the following description of embodiments made with reference to the accompanying drawings in which:

Figure 1 illustrates a graph of absorbance value against concentrations of plant extract for determining the cytotoxicity of *A. cordifolia* (stem) crude extract on L929 fibroblast cells,

Figure 2 is a standard curve using 0.05 to 0.3 mg/ml of glucose solution for determining the total carbohydrate content in *A. cordifolia* (stem) crude extract,

Figure 3 is a standard curve using 0.2 to 1.0 mg/ml of bovine serum albumin solution for determining the total protein content in *A. cordifolia* (stem) crude extract,

Figure 4 is a standard curve using 0.01 to 0.05 mg/ml of cholesterol stock solution for determining the total lipid content in *A. cordifolia* (stem) crude extract,

Figure 5 is a standard curve using 0.5 to 2.5 mg/ml of atropine solution for determining the total alkaloid content in *A. cordifolia* (stem) crude extract,

Figure 6 is a standard curve using 0.2 to 1.0 mg/ml of gallic acid solution for determining the total phenolic content in *A. cordifolia* (stem) crude extract,

Figure 7 is a standard curve using 0.1 to 0.6 mg/ml of rutin solution for determining the total flavonoid content in *A. cordifolia* (stem) crude extract,

Figure 8 is a GC-MS chromatogram shows the retention times for the peaks formed in batch 1 of *A. cordifolia* (stem) crude extract,

Figure 9 is a GC-MS chromatogram shows the retention times for the peaks formed in batch 2 of *A. cordifolia* (stem) crude extract,

Figure 10 is a GC-MS chromatogram shows the structural representation of (E)-9-octadecenoic acid ethyl ester ($C_{20}H_{38}O_2$) in batch 1 extract of *A. cordifolia* (stem) crude extract,

Figure 11 is a GC-MS chromatogram shows the structural representation of (E)-9-octadecenoic acid ethyl ester ($C_{20}H_{38}O_2$) in batch 2 extract of *A. cordifolia* (stem) crude extract,

Figure 12 is a GC-MS chromatogram shows the structural representation of 9,12-octadecadienoic acid ethyl ester ($C_{20}H_{36}O_2$) in batch 1 extract of *A. cordifolia* (stem) crude extract,

Figure 13 is a GC-MS chromatogram shows the structural representation of 9,12-octadecadienoic acid ethyl ester ($C_{20}H_{36}O_2$) in batch 2 extract of *A. cordifolia* (stem) crude extract,

Figures 14A, B and C are photomicrographs of rat aortic explant treated with 1% DMSO, *A. cordifolia* (stem) crude extract and suramin, respectively, for determining the pro-angiogenic activity of *A. cordifolia* (stem) crude extract,

Figure 15 illustrates the absorbance value of L929 mice fibroblast cells treated with different concentrations of *A. cordifolia* (stem) crude extract for determining the proliferation of cells,

Figures 16A to D are images taken on day zero for untreated scratch, and scratches treated with 3.75 mg/ml, 7.5 mg/ml and 15 mg/ml of the *A. cordifolia* crude (stem) extract, respectively,

Figures 16E to H are images taken on the fourth day for untreated scratch, and scratches treated with 3.75 mg/ml, 7.5 mg/ml and 15 mg/ml of the *A. cordifolia* crude (stem) extract, respectively,

Figure 17 is images of wound closure for untreated, membrane treated, 5% *A. cordifolia* (stem) extract treated and 10% *A. cordifolia* (stem) crude extract treated Sprague Dawley adult male rat for determining the pro-angiogenic activity of *A. cordifolia* (stem) crude extract,

Figure 18 is a bar chart shows the wound closure of *A. cordifolia* (stem) crude extract treated wound and untreated wound.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Due to the side effects of synthetic medications for the treatment of wounds, the inventors considered that a plant-derived composition would be the ideal solution as it is highly unlikely that plant resources would result in undesirable side effects when consumed or used medicinally.

Various plants have been previously used for medicinal purposes, although, to date, there has been no effective single plant-derived medicament for the treatment of wounds.

Passiflora edulis from the family Passifloraceae is a plant traditionally known to have anti-microbial, antioxidant and anti-inflammatory capabilities. However, it has not been found to be particularly effective in treating wounds.

On the other hand, the inventors have found that *A. cordifolia*, another member of the family Passifloraceae, is highly effective in the treatment of wounds. This is rather surprising given that it is widely known that parts (roots and fruits) of the *A. cordifolia* plant are in fact poisonous to humans.

Plant resource

Adenia cordifolia (Blume) Engl. (*A. cordifolia*) is a herbaceous to subcoriaceous plant belonging to the family of *Passiflorace*. It is indigenous to Southeast Asian countries and widely distributed in the peat swamp forest. It is also known as *Modecca cordifolia (Blume)*, *Adenia vespertilio (Hallier)*, *Adenia quadrifida (Merr)* and *Adenia populifolia (var)*. It is commonly known as passion fruit. In Malaysia and Indonesia, it is locally known as akar kelipunang and Areuy babaling-bingan, respectively.

This plant is a climber and has woody stems. *A. cordifolia* have tendrils that can be 5 to 7 cm long, with simple primitive leaves. The petioles are usually 1.5 to 2.5 cm long. The leaf blades are membranaceous (9 cm × 7.5 cm × 4 cm) and contain five sets of secondary nerves. Moreover, the base of the blade has a small primitive leaf and the blossoms are bisexual, often large. They consist of five petals and sepals, with five stamens. The ovary is superior with many ovules on each placenta which united with 3 to 5 stigmas. The fruits are yellow with a fleshy endosperm containing plenty of seeds.

The roots and fruits of *A. cordifolia* are known to be poisonous. Medicinal extracts could be obtained from non-poisonous parts of *A. cordifolia* such as its bark, flower, leaves, petioles, and stem.

In the present invention, the inventors have derived the plant extract of this invention from the stem of *A. cordifolia*. When comparing the stem extract with leaf extract, the former shows higher total alkaloid and lipid content which are essential in wound healing.

A. cordifolia crude extract and identification of the compounds in the crude extract

Dried or fresh plant materials may be used for extraction. However, due to the common occurrence of differing water content amongst the sample of plant materials collected, the material is preferably dried before extraction.

To prepare the extract of this invention, the stems of *A. cordifolia* were collected and then dried. Any suitable drying method may be used such as air- or oven-drying. In an embodiment of the present invention, the stems are shade-dried for about 40 days.

In order to increase the efficiency of extraction, plant materials are normally powdered or pulverized to increase surface area before extraction. This will create more surface area for the extraction solvent to penetrate the plant cells and for more effective collection of secondary metabolites.

In an embodiment of the present invention, the dried stems were chopped and ground into a coarse powder form, with particle size of about 2 mm to about 4 mm. Any suitable grinding method for the grinding of plant material may be used. For example, the dried stem may be ground using a suitable commercial blender. The ground stems are kept at a temperature of about 28°C to about 30°C in a sealed environment, prior to the extraction process.

Any suitable extraction process may be used to obtain the crude plant extract. There are generally two methods of extraction for obtaining plant extracts, namely, organic solvent extraction or aqueous extraction. Organic solvents such as chloroform and diethyl ether are suitable for extraction of polar compounds and methanol is suitable for semi-polar compounds. However, these solvents are toxic to humans, not easy to remove and therefore not suitable for use when preparing medicinal plant or herb extracts. Water is a suitable solvent for extracting polar compounds and is not toxic to humans. However, removal of water post-extraction, will need to be conducted at a higher temperature compared to organic solvents, which are more volatile than water. Such a higher process temperature would adversely affect some of the phytocompounds present in the extract, that are less thermally stable.

In an embodiment, the plant extract of this invention was obtained using ethanol as a solvent. Ethanol was selected as it is a universal solvent and is less toxic to human and animal cells. Preferably, 70% ethanol (with the mixture of alcohol and water) is used because it is suitable for the extraction of polar and some non-polar metabolites

in plant material. Furthermore, the temperature and time required to remove 70% ethanol post extraction is lower.

The solvent and water in the resulting extract was removed from the resulting extract to produce a crude plant extract. Any suitable method may be used. In an embodiment of the present invention, a rotary evaporator operating at a temperature of about 40°C was used to remove ethanol followed by a lyophilizer to remove water. Further details are provided in Example 2.

The percentage of viscous crude extract obtained was calculated with the formula below:

$$Y_{extract}\ (\%) = (M_{extract} / m_{feed}) \times 100 \qquad (1)$$

where $Y_{extract}$ is the yield of extraction, $M_{extract}$ is the mass of crude extract (g) and m_{feed} is the mass of feed (g).

It was observed that the yield of crude extraction using this ethanolic extraction method was 8.1%. A sample of 135 g of *A. cordifolia* (stem) crude extract generates 11 g of yield resulting in a percentage of yield of 8.1%.

The medicinal properties of a plant extract are generally attributable to the presence of different phytochemicals. For example, antioxidant activity mainly depends on the presence of phenolics, alkaloids, terpenoids and their derivatives. In this context, it is important to identify the phytochemicals present in the selected plant extract.

The *A. cordifolia* (stem) crude extract of this invention was subjected to gas chromatography mass spectrometry (GC-MS), DNSA method, Folin-Ciocalteu method, colorimetric method and spectrophotometric method to identify the compounds and phytochemicals present in the extract.

The details of the analysis are provided in Examples 4 and 5. The results show that (E)-9-octadecenoic acid ethyl ester ($C_{20}H_{38}O_2$) and 9,12-octadecadienoic acid ethyl

ester ($C_{20}H_{36}O_2$) are predominantly present in the *A. cordifolia* (stem) crude extract of this invention.

The presence of (E)-9-octadecenoic acid ethyl ester ($C_{20}H_{38}O_2$) in an extract is known to indicate anti-inflammatory activity and 9,12-octadecadienoic acid ethyl ester ($C_{20}H_{36}O_2$) (also known as linoleic acid) is known to increase the release of pro-inflammatory mediators in the initial inflammation phase of wound healing and can aid in accelerating wound closure.

Thus, the dominant presence of (E)-9-octadecenoic acid ethyl ester and 9,12-octadecadienoic acid ethyl ester indicates that the *A. cordifolia* (stem) crude extract of this invention has anti-inflammatory activity and is indicated in the treatment of wounds.

The results also show that the *A. cordifolia* (stem) crude extract of this invention comprises phytochemicals such as alkaloids, saponins, phenolics and flavonoids.

It has been known that alkaloids have a wide range of pharmacological activities. For example, alkaloids such as theobromine has antioxidant properties. Similarly, saponins, phenolics and flavonoids have been known to have antioxidant properties. The presence of these phytochemicals further indicates that the *A. cordifolia* (stem) crude extract of this invention has antioxidant activity, another indicator for the treatment of wounds.

When comparing *A. cordifolia* and *Passiflora edulis*, the inventors found that *A. cordifolia* has a higher saponin and phenolic content than *Passiflora edulis*. Further, although (E)-9-octadecenoic acid ethyl ester and 9,12-octadecadienoic acid ethyl ester are found in *A. cordifolia*, it is in fact not present in *Passiflora edulis*. This explains the increased efficacy in wound healing demonstrated by the *A. cordifolia* extract relative to *Passiflora edulis*.

Suitability of the use of *A. cordifolia (stem)* crude extract in the manufacture of a medicament in the treatment of wounds

Cytotoxicity of the A. cordifolia (stem) crude extract

The *A. cordifolia (stem)* crude extract was subjected to a cytotoxicity test with a MTT metabolism-based assay. The assay evaluation of the *A. cordifolia* (stem) crude extract recorded an inhibitory concentration (IC_{50}) of 29.5 mg/ml.

This reveals that 29.5 mg/ml is the optimal concentration for cell proliferation for the *A. cordifolia (stem)* crude extract and that a medicament consisting of the *A. cordifolia (stem)* crude extract at 29.5 mg/ml is safe for human consumption.

Antioxidant activity of the A. cordifolia (stem) crude extract

Oxygen free radicals are toxic waste products which cause oxidative stress during the inflammatory phase of wound healing. Application of antioxidants to wounds have been reported to be effective in scavenging free radicals. Further, antioxidants have also been reported to improve wound healing through increasing collagen cross-linking and breaking strength.

The inventors have confirmed that the *A. cordifolia* (stem) crude extract of this invention is capable of scavenging free radicals, which aid in controlling wound oxidative stress. In Example 6, the free radical scavenging capability of the *A. cordifolia* (stem) crude extract of this invention was investigated with a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

DPPH assay is one of the most common spectrophotometric methods for evaluation of antioxidant activity. This assay is based on the ability of an antioxidant to scavenge stable free radical DPPH, which has a strong purple color and can be measured spectrophotometrically at a wavelength of 517 nm. When scavenged by an antioxidant, the free radical DPPH is reduced to diphenylpicrylhydrazine (DPPHH), whereupon the strong purple colour of the free radical DPPH is converted to pale yellow or colourless. The degree of discoloration indicates the scavenging potential of an antioxidant in terms of the hydrogen donating capability.

The results of DPPH assay are represented as percentage of free radicals scavenging activity calculated with the formula below:

Percentage of free radical scavenging activity = [(Abs.c-Abs.s) /Abs.c] × 100 (2)

where Abs.c is the absorbance of negative control and Abs.s is the absorbance of *A. cordifolia* (stem) crude extract.

The DPPH assay revealed a concentration-dependant antioxidant activity in the *A. cordifolia* (stem) crude extract of this invention.

Pro-angiogenic activity of the A. cordifolia (stem) crude extract

Angiogenesis is a fundamental step in the wound healing process by which new blood vessels are formed from pre-existing vessels. The induction of angiogenesis is attributed to the production of acidic or basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor (VEGF), transforming growth factor beta 1 (TGF-β), angiogenin, angiotropin and angiopoetin-1.

Based on the *ex vivo* study in Example 7, a significant induction of angiogenesis can be observed in the aortic ring treated with the *A. cordifolia* (stem) crude extract of this invention compared to the negative control. The inventors observed that there was significant microvessel development in the *A. cordifolia* (stem) crude extract-treated aortic ring. The growth of microvessels indicates that the *A. cordifolia* (stem) crude extract of this invention has pro-angiogenic activity, which is essential in the treatment of wounds.

Cell proliferative activity of the A. cordifolia (stem) crude extract

In wound healing, the proliferative phase begins on the third day of injury and normally persists for about two weeks. During proliferation, the wound is rebuilt with new granulation tissue which consists of collagen and extracellular matrix. The wound contracts as new tissues are built. In addition, a new network of blood vessels must be constructed so that the granulation tissue can be healthy and receive

sufficient oxygen and nutrients. Myofibroblast cells cause the wound to contract by gripping the wound edges and pulling them together.

In example 8, an *in vitro* study was conducted to assess the effect of the *A. cordifolia* (stem) crude extract of this invention on L929 mice fibroblast cells with minor modification by using MTT assay. MTT assay is a quantitative detection of cell proliferation as it measures the growth rate of cells by virtue of a linear relationship between cell activity and absorbance value. The result shows that the proliferation of L929 mice fibroblast cells increases as the concentration of the *A. cordifolia* (stem) crude extract increases.

The increase of cell proliferation in the *A. cordifolia* (stem) crude extract treated fibroblast cells indicates that the crude extract of this invention has the capacity to promote cell proliferation, which is again essential in the treatment of wounds.

In example 9, the wound healing property of the *A. cordifolia* (stem) crude extract was evaluated by its ability to stimulate wound closure in *in vitro* grown fibroblast cells. The result of the scratch assay shows that the wound treated with the *A. cordifolia* (stem) crude extract of this invention exhibits a significantly higher area of wound closure. This indicates that the crude extract has the capability to stimulate the proliferation of fibroblast cells, which are required for wound closure.

Epithelialization is an essential component of wound healing and is generally used as a defining parameter of successful wound closure. Epithelial cells begin to migrate from the wound area within a few hours of injury and the process lasts until a single layer of cells are formed over the wound area. As the mitotic activity of epithelial cells increases, the migrating cells form a complete layer over the wound and attach to cell matrix. This process completes when the matured epithelial cells assemble thus resulting in the formation of basement membrane.

Based on the *in vivo* study in example 10, a wound treated with the *A. cordifolia* (stem) crude extract of this invention exhibits a significantly higher area of wound closure. Again, this indicates that the *A. cordifolia* (stem) crude extract of this

invention has the ability to stimulate wound closure and is suitable to be used as a natural plant-based medicine in healing wounds.

Use of the *A. cordifolia* (stem) crude extract in the manufacture of a medicament in the treatment of wounds

The present invention provides a novel use of an extract derived from the stem of *A. cordifolia* in the manufacture of a medicament for administration to a human in the treatment of wounds.

The extract may be processed into any suitable orally acceptable medicinal dosage form such as pellets, tablets, capsules, powders, granules, emulsions or suspensions.

The medicament may contain any other suitable additives or carriers for various purposes such as to facilitate absorption, to prolong shelf-life and stability of the medicament, to reduce viscosity, to enhance solubility, etc. For example, a glidant such as corn starch may be added to the medicament in powder form to improve its flowability. If the medicament is in capsule form, corn starch, a natural diluent may again be added. If an oral suspension is desired, a flavour enhancer may be added to the medicament to mask the taste of the medicament.

The extract may also be administrated topically such as in the form of creams, gels, lotions, powders, foams, ointments or transdermal patches. The topical medicament may also include suitable additives or carriers. For example, natural carriers such as vegetable or mineral oils, may be added to creams, gels or lotions. For transdermal patches, a penetration enhancer may be added, as would generally be practiced.

The administration route and dosage regime of a medicament of this invention is largely dependent on the type of wounds to be treated such as acute or chronic wounds, open or closed wounds and the severity of the wound. Apart from the type of wounds, the age, gender, body size and medical condition of the patient in need

of the medicament are also factors when prescribing the *A. cordifolia* (stem) crude extract medicament of this invention.

For example, diabetic foot ulcer is the most common foot injury that leads to lower extremity amputation in diabetics and is a major complication of diabetes mellitus. As known, diabetes mellitus is a metabolic disorder that impedes the normal steps of the wound healing process, including homeostasis, inflammation, matrix deposition and angiogenesis. The expression of growth factors which are required to stimulate angiogenesis are relatively low at a diabetic ulcer site.

Since the natural healing mechanism cannot be self-initiated by a diabetic patient, the medicament of this invention would need to be administered to diabetics at a higher dosage compared to a non-diabetic patient, to stimulate angiogenesis and promote wound healing. The medicament of this invention could be topically applied directly to the ulcer site. When necessary, the medicament could also be ingested orally. Further, the high level of blood glucose in diabetics slows down the ulcer healing process. Thus, a longer total time of treatment may be required compared to a non-diabetic patient.

The medicament of this invention may also be used both orally and topically, concurrently. For example, in the treatment of chronic diabetic foot ulcer or a mouth ulcer.

EXAMPLES

The following Examples illustrate the various aspects, and methods and steps for obtaining the extract of this invention and identifying the phytochemical properties of said extract. These Examples do not limit the invention, the scope of which is set out in the appended claims.

Example 1

Preparation of *A. cordifolia* material for extraction

The stems of *A. cordifolia* was collected from Kwang & Sons, Cameron Highlands at 1200 m altitude, latitude: 4° 31' 26.99" and longitude: 101° 20' 12.00" E.

Identification of the plant was carried out by Forest Research Institute Malaysia (FRIM). The stems of *A. cordifolia* were shade-dried; chopped and weighed using a weighing balance. The chopped stems were later ground to a coarse powder using a commercial blender.

Example 2

Ethanol extraction of *A. cordifolia* material

135 g of the coarse powder was extracted consecutively with 70% ethanol using a Soxhlet apparatus heater assembly for 17 hours at 40°C. The extract was concentrated from 900 ml to 300 ml using a rotary evaporator at 80 rpm and at a temperature of 40°C. The concentrated extract was frozen in a -80°C freezer and subsequently lyophilized for 48 hours using a freeze dryer machine under vacuum condition, at of pressure of 150 Pa and at a temperature of -47°C. The lyophilized *A. cordifolia* extract was stored at room temperature until further use.

The result of the extraction is shown in Table 1.

Table 1: Methanol extraction of *A. cordifolia* material

Cycles	Mass of initial feed (g)	Volume of extract (ml)	Mass of final extract (g)	Ratio (Feed: Extract)
26	75	500	6	12.1
	60	400	5	12.1
Total	135	900	11	12.1

Example 3

Cytotoxicity test of *A. cordifolia* (stem) crude extract

L929 mice fibroblast cells of passage no. 21 was revived from cell repository of QIUP Biotechnology Laboratory. Three vials of fibroblast cells were thawed and transferred into a falcon tube. 3 ml of FCS and 25 ml of DMEM with phenol red were added, and the fibroblast cells were centrifuged at 300 g for 5 minutes using a centrifuge. The supernatant was decanted and pellet was resuspended with Dulbecco's Phosphate Buffer Saline (DPBS) and centrifuged at 300 g for 5 minutes. Following centrifugation,

the supernatant was decanted. The fibroblast cells were resuspended with 8 ml of DMEM with phenol red. About 2 µl of fibroblast cells were seeded in a 6-well plate and 3 µl of cells were seeded in a T-25 flask. Later, DMEM with phenol red growth media was added and the 6-well plate and T-25 flask were incubated in a 5% CO_2 incubator at 37°C. The fibroblast cells were monitored regularly using inverted and bright field microscope for formation of 100% confluent cells monolayer and the fibroblast cells were sub-cultured periodically to maintain the availability of the cell line.

0.1 g of *A. cordifolia* (stem) crude extract was dissolved in 10 ml of distilled water. The mixture was vortexed until the extract was fully dissolved in distilled water.

The protocol of cytotoxicity was adopted from CellTiter 96® Non-Radioactive Cell Proliferation Assay manufactured by Promega using fibroblast cells. Each sample, including a negative control without *A. cordifolia* (stem) crude extract, was assayed in triplicate. Various concentrations of *A. cordifolia* (stem) crude extract was prepared by diluting 60 mg of the extract in 1 ml of DMEM-5% FCS media. The crude extract was prepared by two-fold dilution and four various concentrations of extract were prepared for this assay.

About 100 µl of L929 fibroblast cells were seeded into a 96-well plate. 50 µl of *A. cordifolia* (stem) crude extract with concentration ranging from 7.5 mg/ml, 15 mg/ml, 30 mg/ml, and 60 mg/ml were added to each well and the plate was incubated at 37°C in a humidified, 5% CO_2 incubator for 24 hours. About 15 µl of Dye Solution was added to each well after 24 hours. The assay plate was covered with aluminium foil and incubated at 37°C for 4 hours in a humidified, 5% CO_2 incubator.

After incubation, the formation of formazan crystals was photographed using an inverted phase contrast microscope. Later, 100 µl of the solubilization solution was added to each well. The plate was incubated for one hour and then the content of the wells was mixed to get a uniformly colored solution. The absorbance value of each well was taken at wavelength 570 nm using multiwell plate and the results is shown in Table 2.

Table 2: Absorbance value for cytotoxicity of *A. cordifolia* (stem) crude extract on L929 mice fibroblast cells

Concentration (mg/ml)	Absorbance
60	0.082
30	0.043
15	0.030
7.5	0.022

The results in Table 2 show a linear relationship between the concentration of *A. cordifolia* (stem) crude extract of this invention and absorbance value, namely, the proliferation of cell increases when the concentration of the *A. cordifolia* (stem) crude extract increases. A line graph (as shown in Figure 1) was plotted based on the results in the Table 2 to identify the IC_{50} value. It shows that 29.5 mg/ml is the optimal concentration for cell proliferation for the *A. cordifolia (stem)* crude extract and that a medicament consisting of the *A. cordifolia (stem)* crude extract at 29.5 mg/ml is safe for human consumption.

Example 4

Phytochemical analysis of *A. cordifolia* (stem) crude extract

The *A. cordifolia* (stem) crude extract at different concentrations were subjected to a phytochemical analysis. The test was repeated for 5 batches of the *A. cordifolia* (stem) crude extract.

Determination of total carbohydrate content

3,5-dinitrosalicyclicacid (DNSA) method was used to determine the total carbohydrate content of *A. cordifolia* (stem) crude extract.

About 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 ml of the *A. cordifolia* (stem) crude extract were added into each test tube containing 0.95, 0.90, 0.85, 0.80, 0.75 and 0.70 ml of distilled water and 2 ml of DNSA reagent, respectively. The mixtures were heated in a boiling water bath for 5 minutes. Then, the test tubes were cooled to room temperature. The mixture in each test tube was diluted with 7 ml of distilled water and mixed well.

The absorbance of the mixtures was measured at 540 nm using a spectrophotometer and recorded. A standard curve was plotted for the data recorded, as shown in Figure 2.

The results in Table 3 show the presence of carbohydrate in *A. cordifolia* (stem) crude extract. The carbohydrate content in each batch of *A. cordifolia* (stem) crude extract was not significantly different as the ($p > 0.05$). Further, the presence of carbohydrate was constant when the concentration of *A. cordifolia* (stem) crude extract increases.

Table 3: Total carbohydrate contents of *A. cordifolia* (stem) crude extract

Concentration of carbohydrates (GE/g)					
Batch	Concentration of *A. cordifolia* (stem) crude extract (mg/ml)				
	0.05	0.10	0.15	0.20	0.25
1	0.053 ± 0.003^{e}	0.092 ± 0.003^{d}	0.174 ± 0.004^{c}	0.379 ± 0.005^{b}	0.762 ± 0.007^{a}
2	0.051 ± 0.002^{e}	0.096 ± 0.001^{d}	0.185 ± 0.005^{c}	0.387 ± 0.003^{b}	0.766 ± 0.001^{a}
3	0.052 ± 0.002^{e}	0.095 ± 0.001^{d}	0.190 ± 0.003^{c}	0.386 ± 0.003^{b}	0.766 ± 0.003^{a}
4	0.054 ± 0.003^{e}	0.108 ± 0.006^{d}	0.191 ± 0.002^{c}	0.391 ± 0.002^{b}	0.774 ± 0.003^{a}
5	0.051 ± 0.002^{e}	0.099 ± 0.003^{d}	0.198 ± 0.002^{c}	0.388 ± 0.003^{b}	0.770 ± 0.005^{a}
Average	0.052 ± 0.002^{e}	0.098 ± 0.007^{d}	0.189 ± 0.003^{c}	0.386 ± 0.003^{b}	0.768 ± 0.004^{a}

The values are mean ± standard error mean (n=3); GE: Glucose Equivalent;
Values with different superscript in a row differ significantly ($p < 0.05$).

Determination of total protein content

The Folin-Ciocalteu method was used to determine the total protein content of *A. cordifolia* (stem) crude extract.

The reaction mixture contains 0.2, 0.4, 0.6, 0.8 and 1.0 ml of *A. cordifolia* (stem) crude extract and 1.8, 1.6, 1.4, 1.2 and 1.0 ml of distilled water in each of the test tubes. From these different dilutions, 0.2 ml of the reaction mixture was pipetted out to different test tubes. Then, 2 ml of alkaline copper sulphate reagent, $CuSO_4$ was added and mixed well. The solutions were incubated at room temperature for 10 minutes. After 10 minutes, 0.2 ml of Folin-Ciocalteu reagent was added to each

test tube and incubated for 30 minutes. The absorbance for the mixtures was determined against the reagent blank at 660 nm with a spectrophotometer and recorded. A standard curve was plotted for the data recorded, as shown in Figure 3.

The results in Table 4 show the presence of protein in *A. cordifolia* (stem) crude extract. There was a significant increase in the content of protein when the concentration of *A. cordifolia* (stem) crude extract increases.

Table 4: Total protein contents of *A. cordifolia* (stem) crude extract

Concentration of proteins (BSAE/g)					
Batch	Concentration of *A. cordifolia* (stem) crude extract (mg/ml)				
	0.2	0.4	0.6	0.8	1.0
1	0.175 ± 0.004^{e}	0.348 ± 0.002^{d}	0.677 ± 0.003^{c}	1.348 ± 0.002^{b}	2.951 ±0.040^{a}
2	0.199 ± 0.01^{e}	0.409 ± 0.005^{d}	0.805 ± 0.015^{c}	1.613 ± 0.000^{b}	3.112± 0.082^{a}
3	0.198 ± 0.009^{e}	0.352 ± 0.002^{d}	0.830 ± 0.002^{c}	1.706 ± 0.009^{b}	3.134 ± 0.063^{a}
4	0.182 ± 0.005^{e}	0.336 ± 0.005^{d}	0.825 ± 0.003^{c}	1.728 ± 0.003^{b}	3.244 ± 0.008^{a}
5	0.198 ± 0.003^{e}	0.319 ± 0.002^{d}	0.733 ± 0.006^{c}	1.778 ± 0.014^{b}	3.316 ± 0.006^{a}
Average	0.190 ± 0.006^{e}	0.353 ± 0.003^{d}	0.774 ± 0.006^{c}	1.635 ± 0.006^{b}	3.151 ± 0.040^{a}

The values are mean ± standard error mean (n=3);

BSAE: Bovine Serum Albumin Equivalent;

Values with different superscript in a row differ significantly ($p < 0.05$).

Determination of total lipid content

The colorimetric method was used to determine the total lipid content of the *A. cordifolia* (stem) crude extract.

Each test tube contains 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the *A. cordifolia* (stem) crude extract and 1.8, 1.6, 1.4, 1.2 and 1.0 ml of ethanol, respectively. 2 ml of acid-ferric chloride reagent was added into a blank and into each of the reaction mixtures in the test tubes. The mixtures were mixed using a vortex mixer and incubated for 30 minutes at room temperature. The absorbance for the solutions was determined

against the reagent blank at 660 nm with an UV spectrophotometer and recorded. A standard curve was plotted for the data recorded, as shown in Figure 4.

The results in Table 5 show the presence of lipid in the *A. cordifolia* (stem) crude extract. There was a significant increase in the content of lipid when the concentration of *A. cordifolia* (stem) crude extract increases.

Table 5: Total lipid contents of *A. cordifolia* (stem) crude extract

Concentration of lipids (CSE/g)					
Batch	Concentration of *A. cordifolia* (stem) crude extract (mg/ml)				
	0.01	0.02	0.03	0.04	0.05
1	0.020 ± 0.003^{e}	0.046 ± 0.001^{d}	0.105 ± 0.003^{c}	0.212 ± 0.005^{b}	0.408 ± 0.005^{a}
2	0.025 ± 0.002^{e}	0.049 ± 0.004^{d}	0.107 ± 0.004^{c}	0.205 ± 0.003^{b}	0.392 ± 0.004^{a}
3	0.025 ± 0.002^{e}	0.050 ± 0.002^{d}	0.102 ± 0.002^{c}	0.202 ± 0.003^{b}	0.398 ± 0.005^{a}
4	0.033 ± 0.001^{e}	0.053 ± 0.003^{d}	0.111 ± 0.002^{c}	0.218 ± 0.008^{b}	0.410 ± 0.003^{a}
5	0.028 ± 0.002^{e}	0.062 ± 0.004^{d}	0.115 ± 0.002^{c}	0.220 ± 0.002^{b}	0.416 ± 0.008^{a}
Average	0.026 ± 0.002^{e}	0.052 ± 0.003^{d}	0.108 ± 0.003^{c}	0.211 ± 0.004^{b}	0.405 ± 0.005^{a}

The values are mean ± standard error mean (n=3); CSE: Cholesterol Equivalent; Values with different superscript in a row differ significantly ($p < 0.05$).

Determination of total alkaloid content

The spectrophotometric method was used to determine the total alkaloid content of *A. cordifolia* (stem) crude extract.

0.5, 1.0, 1.5, 2.0 and 2.5 ml of *A. cordifolia* (stem) crude extract were measured and added into each test tube separately. 5 ml of phosphate buffer (pH 4.7) and 5 ml of bromocresol green (BCG) solution were added to each of the test tubes. These solutions were shaken vigorously along with 4 ml of chloroform. The absorbance of the mixtures in chloroform was measured at 470 nm against the blank prepared above without atropine with an UV/Visible spectrophotometer and recorded. A standard curve was plotted for the data recorded, as shown in Figure 5.

The results in Table 6 show the presence of alkaloid in the *A. cordifolia* (stem) crude extract. There was an increase in the content of alkaloid when the concentration of *A. cordifolia* (stem) crude extract increases.

Table 6: Total alkaloid contents of *A. cordifolia* (stem) crude extract

Concentration of alkaloids (AE/g)					
Batch	Concentration of *A. cordifolia* (stem) crude extract (mg/ml)				
	0.2	0.4	0.6	0.8	1.0
1	0.304 ± 0.031^{e}	0.611 ± 0.003^{d}	1.222 ± 0.004^{c}	2.428 ± 0.044^{b}	4.828 ± 0.015^{a}
2	0.313 ± 0.004^{e}	0.614 ± 0.013^{d}	1.220 ± 0.005^{c}	2.453 ± 0.009^{b}	4.952 ± 0.009^{a}
3	0.264 ± 0.003^{e}	0.551 ± 0.009^{d}	1.191 ± 0.017^{c}	2.383 ± 0.009^{b}	4.895 ± 0.034^{a}
4	0.360 ± 0.007^{e}	0.634 ± 0.007^{d}	1.226 ± 0.004^{c}	2.513 ± 0.025^{b}	4.903 ± 0.047^{a}
5	0.272 ± 0.007^{e}	0.567 ± 0.005^{d}	1.195 ± 0.009^{c}	2.382 ± 0.045^{b}	4.825 ± 0.022^{a}
Average	0.303 ± 0.010^{e}	0.595 ± 0.007^{d}	1.211 ± 0.008^{c}	2.432 ± 0.026^{b}	4.881 ± 0.025^{a}

The values are mean ± standard error mean (n=3); AE: Atropine Equivalent;
Values with different superscript in a row differ significantly ($p < 0.05$).

The presence of alkaloids indicates that the *A. cordifolia* (stem) crude extract of this invention has antioxidant activity which is essential for wound healing.

Determination of total saponin content

Various amounts of *A. cordifolia* (stem) crude extracts were prepared in different amount of solutions. About 1 and 2 g of *A. cordifolia* (stem) crude extract was diffused in 10 and 20 ml of 20% ethanol, respectively. The mixture was then heated in a hot water bath at 55°C for 4 hours with continuous stirring. After that, the mixture was vacuum filtered and the residue obtained after vacuum filtration was re-extracted again with 10 and 20 ml of 20 % ethanol and decreased to 4 and 6 ml over hot water bath at 100°C. The concentrated solution was mixed vigorously with 2 and 6ml of diethyl ether each in a separating funnel. About 4 and 8 ml butanol was added into the collected aqueous layer. It was then washed with 2 and 6 ml of 5% w/v aqueous sodium chloride. The whole mixture was heated in a hot water bath and later oven dried at 40°C to a constant weight.

The results in Table 7 show the presence of saponin in the *A. cordifolia* (stem) crude extract. The final constant weight of saponin increases as the weight of *A. cordifolia* (stem) crude extract increases.

Table 7: Total saponin contents of *A. cordifolia* (stem) crude extract

Final constant weight of saponin		
Batch	Weight of *A. cordifolia* (stem) crude extract (g)	
	1	2
1	0.113 ± 0.003^{b}	0.203 ± 0.007^{a}
2	0.110 ± 0.006^{b}	0.197 ± 0.009^{a}
3	0.113 ± 0.009^{b}	0.193 ± 0.003^{a}
4	0.110 ± 0.012^{b}	0.193 ± 0.009^{a}
5	0.100 ± 0.006^{b}	0.210 ± 0.006^{a}
Average	0.109 ± 0.007^{b}	0.199 ± 0.007^{a}

The values are mean ± standard error mean (n=3);

Values with different superscript in a row differ significantly ($p < 0.05$).

The presence of saponin indicates that the *A. cordifolia* (stem) crude extract of this invention has antioxidant activity which is essential for wound healing.

Determination of total phenolic content

7.5% sodium carbonate (Na_2CO_3) was prepared by adding 7.5 g of Na_2CO_3 (MW 105.99 g/mol) in distilled water that made up 100 ml.

About 1 mg/ml of *A. cordifolia* (stem) crude extract in DMSO (1 mg/ml) was diluted to 0.10, 0.20, 0.40, 0.60, 0.80 and 1.00 mg/ml in test tubes, respectively. Then, about 800 µl of Folin-Ciocalteu reagent and 2ml of 7.5% Na_2CO_3 were added to each of the test tubes. These mixtures were further diluted to 7ml/mg with distilled water and were incubated at 24°C in the dark for 2 hours. The absorbance of each mixture was measured at a wavelength of 765 nm using a spectrophotometer and recorded. A standard curve was plotted for the data recorded, as shown in Figure 6.

The results in Table 8 show the presence of phenolic in the *A. cordifolia* (stem) crude extract. There was an increase in the content of phenolic when the concentration of *A. cordifolia* (stem) crude extract increases.

Table 8: Total phenolic contents of *A. cordifolia* (stem) crude extract

Concentration of phenolics (GAE/g)						
Batch	Concentration of *A. cordifolia* (stem) crude extract (mg/ml)					
	0.0	0.2	0.4	0.6	0.8	1.0
1	0.000	0.121	0.221	0.316	0.448	0.503
2	0.000	0.119	0.218	0.319	0.445	0.507
3	0.000	0.120	0.223	0.320	0.451	0.502
Average	0.000	0.120	0.221	0.318	0.448	0.504

The presence of phenolics indicates that the *A. cordifolia* (stem) crude extract of this invention has antioxidant activity which is essential for wound healing.

Determination of total flavonoid content

20% aluminum trichloride ($AlCl_3$) was prepared by adding 20 g of $AlCl_3$ in distilled water that made up 100 ml.

About 0.01 g of *A. cordifolia* (stem) crude extract in 10 ml of methanol (1 mg/ml) was diluted to 0.10, 0.20, 0.30, 0.40 and 0.50 mg/ml in test tubes. Then, 100 µl of 20% $AlCl_3$ in CH_3OH and a drop of acetic acid were added to each of the test tubes and each tube was made up to a final volume of 5 ml by methanol. The mixtures were incubated at 24°C for 40 minutes. The absorbance of each mixture was measured at a wavelength of 415 nm using a spectrophotometer and recorded. A standard curve was plotted for the data recorded, as shown in Figure 7.

The results in Table 9 show the presence of flavonoid in the *A. cordifolia* (stem) crude extract. There was an increase in the content of flavonoid when the concentration of *A. cordifolia* (stem) crude extract increases.

Table 9: Total flavonoid contents of *A. cordifolia* (stem) crude extract

Concentration of flavonoid (RE/g)							
Batch	Concentration of *A. cordifolia* (stem) crude extract (mg/ml)						
	0.0	0.1	0.2	0.3	0.4	0.5	0.6
1	0.000	0.059	0.120	0.187	0.227	0.302	0.351
2	0.000	0.061	0.119	0.188	0.229	0.329	0.353
3	0.000	0.063	0.118	0.186	0.230	0.301	0.349
Average	0.000	0.061	0.119	0.187	0.229	0.311	0.351

The presence of flavonoids indicates that the *A. cordifolia* (stem) crude extract of this invention has antioxidant activity which is essential for wound healing.

Table 10: Summary result of phytochemical screening tests

Phytocontents	Average contents in 1 mg/ml *A. cordifolia* (stem) crude extract	Contents in 30 mg/ml *A. cordifolia* (stem) crude extract (µg)
Carbohydrate	3.072 ± 0.016	92.16 ± 0.480
Protein	3.151 ± 0.040	94.53 ± 1.200
Lipid	8.100 ± 0.100	243.00 ± 3.000
Alkaloid	4.881 ± 0.025	146.40 ± 0.750
Saponin	0.109 ± 0.007	3.27 ± 0.210
Phenolic	0.504± 0.003	15.12 ± 0.090
Flavonoid	6.117 ± 0.032	183.5 ± 0.953

From Table 10, 30 mg/ml of *A. cordifolia* extracted from the stem of the plant comprises 92.16 ± 0.480 µg of carbohydrate, 94.53 ± 1.200 µg of protein, 243.0 ± 3.000 µg of lipid, 146.4 ± 0.750 µg of alkaloid, 3.27 ± 0.210 µg of saponin, 15.12 ± 0.090 µg of phenolic and 183.5 ± 0.953 µg of flavonoid.

The presence of alkaloids, saponins, phenolics and flavonoids indicates that the *A. cordifolia* (stem) crude extract of this invention has antioxidant activity which is essential for wound healing.

Example 5
Gas Chromatography-Mass Spectrometry (GC-MS) analysis of *A. cordifolia* (stem) crude extract

The GC-MS analyses for the *A. cordifolia* (stem) crude extract was carried out using Gas Chromatography (GC) Thermo Trace 1300 and Triple Quadrupole MS Thermo TSQ 8000 equipment. Helium gas was used as the carrier gas and the carrier mode was a constant flow at 1.5 ml/minute. The temperature programming was set with initial oven temperature at 40°C, held for 3 minutes and the final temperature of oven was 250°C. 1 µl of *A. cordifolia* (stem) crude extract was injected into the GC-MS using split mode (50). The split flow was 75 ml/minute. The total running time for batch 1 and batch 2 of the *A. cordifolia* (stem) crude extract were 31.11 and 31.13 minutes. Relative quantitative determinations were made by relating respective peak areas to total ion chromatogram (TIC).

According to the results shown in Figures 8 and 9, the retention times for the peaks formed in batch 1 extraction are 35.41 and 35.51 minutes, whereas the retention times for peaks formed in batch 2 extraction are 35.42 and 35.52 minutes, respectively. Thus, 35.41/35.42 minutes and 35.51/35.52 minutes retention times might be considered reference values for the *A. cordifolia* (stem) crude extract.

Further, the results of GC-MS show that (E)-9-octadecenoic acid ethyl ester ($C_{20}H_{38}O_2$) and 9,12-octadecadienoic acid ethyl ester ($C_{20}H_{36}O_2$) are the predominant compounds present in batch 1 and 2 extracts of the *A. cordifolia* (stem) crude extract. Figures 10 and 11 show the structural representation of (E)-9-octadecenoic acid ethyl ester in batch 1 and 2 extracts of the *A. cordifolia* (stem) crude extract, respectively. Figures 12 and 13 show the structural representation of 9,12-octadecadienoic acid ethyl ester in batch 1 and 2 extracts of the *A. cordifolia* (stem) crude extract, respectively.

The dominant presence of (E)-9-octadecenoic acid ethyl ester and 9,12-octadecadienoic acid ethyl ester indicates that the *A. cordifolia* (stem) crude extract of this invention has anti-inflammatory activity and is indicated in the treatment of wounds.

Example 6

Antioxidant test of *A. cordifolia* (stem) crude extract

Various concentration of the *A. cordifolia* (stem) crude extract was prepared by diluting 2 mg of the extract in 1 ml of methanol. The extract was prepared by two-fold dilution and ten various concentrations of 1.25 µg/ml, 2.5 µg/ml, 5.00 µg/ml, 10.00 µg/ml, 20.00 µg/ml, 40.00 µg/ml, 80.00 µg/ml, 160.00 µg/ml, 320.00 µg/ml and 640 µg/ml were prepared for this assay.

A positive control was prepared by adding 50 µl (5 mg/mL) of L-ascorbic acid and 450 µl of Tri-HCl buffer in a sterile Eppendorf tube. A negative control was prepared by adding 50 µl of methanol and 450 µl of Tris-HCl buffer in another sterile Eppendorf tube.

The reaction mixtures were prepared by adding 50 µl of the *A. cordifolia* (stem) crude extract with different concentrations and 450 µl of Tris-HCl buffer for each sample. About 1000 µl of DPPH solution was added to all the Eppendorf tubes and the reaction mixtures were incubated in dark for 30 minutes. The absorbance of each mixture was measured at a wavelength of 517 nm using a spectrophotometer and the percentage of radical scavenging activity of the *A. cordifolia* (stem) crude extract were shown in Table 11.

Table 11: Percentage of free radical scavenging activity of *A. cordifolia* (stem) crude extract

Treatment	Concentrations (µg/ml)	Percentage of free radical scavenging activity (%)
		Mean ± standard error of mean
Negative control	-	0.00 ± 0.00^{i}
A. cordifolia (stem) crude extract	1.25	5.21 ± 1.014^{i}
	2.50	12.58 ± 0.870^{h}
	5.00	17.96 ± 0.499^{g}
	10.00	23.53 ± 0.123^{f}

	20.00	30.56 ± 0.163^{e}
	40.00	35.80 ± 0.748^{d}
	80.00	40.49 ± 0.048^{c}
	160.00	47.20 ± 0.061^{b}
	320.00	**50.07 ± 0.792^{b}**
	640.00	63.65 ± 0.695^{a}
Positive control (Ascorbic acid)	1.25	33.50 ± 0.976^{i}
	2.50	39.63 ± 1.131^{h}
	5.00	**50.36 ± 0.456^{g}**
	10.00	55.96 ± 0.600^{f}
	20.00	61.95 ± 0.851^{e}
	40.00	70.61 ± 0.114^{d}
	80.00	74.45 ± 0.120^{c}
	160.00	80.85 ± 0.207^{b}
	320.00	86.76 ± 0.251^{a}
	640.00	89.39 ± 0.296^{a}

The result shows a concentration-dependant antioxidant activity in the *A. cordifolia* (stem) crude extract of this invention, namely, the percentage of free radical scavenging activity increases when the concentration of the *A. cordifolia* (stem) crude extract increases. The highest percentage of free radical scavenging activity of the *A. cordifolia* (stem) crude extract at 640.00 µg/ml is 63.65 ± 0.695 ^{a}, whereas of ascorbic acid at 640.00 µg/ml is 89.39 ± 0.296^{a}.This indicates that the *A. cordifolia* (stem) crude extract has antioxidant activity, which is essential for wound healing.

The inhibitory concentration (IC_{50}) value of the *A. cordifolia* (stem) crude extract of this invention is determined at the concentration of 320 µg/ml, where the percentage of free radical scavenging activity is 50.07 ± 0.792 SEM. The IC_{50} value of ascorbic acid is determined at the concentration of 5 µg/ml, where the percentage of free radical scavenging activity is 50.36 ± 0.456 SEM. The result exhibits that the *A. cordifolia* (stem) crude extract of this invention has the capability to scavenge free radical in DPPH assay, which is essential in scavenging free radical in the treatment of wounds.

radical in DPPH assay, which is essential in scavenging free radical in the treatment of wounds.

Example 7

***Ex vivo* study of pro-angiogenic activity of *A. cordifolia* (stem) crude extract**

Thoracic aortas were removed from euthanized male Sprague Dawley (12–14 weeks old), rinsed with serum free medium and cleansed of fibro-adipose tissues. The aortas were cross sectioned into small rings (approximately 1 mm thickness) and seeded individually in 48-wells plate in 300 µL serum free M199 media containing 3 mg/ml fibrinogen and 5 mg/ml aprotinin. 10 µL of thrombin was added into each well and incubated at 37°C for 90 min to solidify.

A second layer of M 199 medium supplemented with 20% HIFBS, 0.1% ε-aminocaproic acid, 1% L-Glutamine, 2.5 µg/ml amphotericin B, and 60 µg/ml gentamicin was added into each well (300 µL/well). 100 µg/ml of the *A. cordifolia* (stem) crude extract was added. Suramin of 100 µg/ml and DMSO 0.1% were used as positive and negative controls, respectively. On day four, the medium was replaced with a fresh one containing the test materials. On day five, the aortic rings were photographed using AMG EVOS fluorescent inverted microscope (40x magnification) as shown in Figure 14 and subsequently the length of blood vessel outgrowth from the primary tissue explants was measured using Leica Quin software.

The percentage of blood inhibition was calculated using the following formula:

$$\text{Percentage (\%) blood vessels inhibition} = [1-(A0/A)] \times 100 \quad (3)$$

where A0 = distance of blood vessels growth in treated rings in µm and A = distance of blood vessels growth in the control in µm.

Table 12: Result of the blood vessels growth

Distance of blood vessels growth (µm)		
A0		A
Suramin treated ring	*A. cordifolia* (stem) crude extract treated aortic ring	Untreated ring
53.5	801.1	677.2

The result shows that 118±7 % of vessel growth in the *A. cordifolia* (stem) crude extract treated aortic ring while 92.1±3 % of vessel inhibition in the suramin treated aortic ring.

As can be seen in the photomicrographs in Figure 14, the aortic ring treated with the *A. cordifolia* (stem) crude extract of this invention shows significant growth of microvessels (Figure 14B) compared to the aortic rings treated with only suramin (Figure 14C) and DMSO 0.1% (Figure 14A). The growth of microvessels indicates that the *A. cordifolia* (stem) crude extract of this invention has pro-angiogenic activity, which is essential in the treatment of wounds.

Example 8

In vitro study of *A. cordifolia* (stem) crude extract in fibroblast cell proliferation - MTT assay

The MTT assay was used to assess the effect of the *A. cordifolia* (stem) crude extract on L929 mice fibroblast cells with minor modification. Mice fibroblast cells were seeded at density of 2×10^5 in a 96 wells flat-bottomed plate. After 24 hours, the *A. cordifolia* (stem) crude extract was added to each well that contains the fibroblast cells at different concentrations of 7.5, 15, 30 and 60 mg/mL. The plate was incubated at 37°C in an incubator under 5% CO_2. After 48 hours, 20 µL of MTT reagent was added to each of the wells as an indicator of cell viability. The assay plates were read using microplate reader at 570 nm absorbance with a reference wavelength of 620 nm. Data were recorded as shown in Table 13 and analyzed for the assessment of the effects of test substance on cell (XpertTM MTT Cell Assay Teaching Kit).

Table 13: Absorbance value of L929 mice fibroblast cells treated with different concentrations of *A. cordifolia* (stem) crude extract

Concentration (mg/ml)	Absorbance
Positive control	0.015
7.5	0.022
15	0.030
30	0.037
60	0.048

The result shows that the fibroblast cells proliferation increases as the concentration of the *A. cordifolia* (stem) crude extract of this invention increases. This indicates that the *A. cordifolia* (stem) crude extract of this invention has proliferative activity, which is essential in the treatment of wounds.

Example 9

In vitro study of _A. cordifolia_ (stem) crude extract in fibroblast cell proliferation – scratch assay

L929 mice fibroblast cell was subjected to cell proliferation study with a scratch assay. The cell monolayer was scraped in a straight line using a p200 pipette tip in order to create a "scratch". The debris was removed, and the edge of the scratch was smoothened by washing the cells once with 1 ml of the growth medium. Then, 3 ml of growth media was added to the control well (without *A. cordifolia* extract) and other wells with the scratched fibroblast cells. Next, 100 µL of various concentrations of the *A. cordifolia* (stem) crude extract of 3.75, 7.5 and 15 mg/ml were added to each well (except control well) to assess its effect in proliferating the scratched fibroblast cells.

Reference points were made close to the scratch by etching the well plate lightly with a razor blade on the outer bottom of the dish to obtain the same field during the image acquisition. After the reference points were made, the well plate was placed under a phase-contrast microscope (OLYMPUS CKX41) and the reference

Example 10

In vivo pro-angiogenic study of *A. cordifolia* (stem) crude extract

The *in vivo* wound closure was investigated using Sprague Dawley adult male rats weighing between 180 and 200 gm. The rats were anesthetized with ketamine/xylazine (100 and 20 mg/kg, ip, respectively). Their skin were shaved with an electrical shaver, disinfected with 70% alcohol and the area on the back of the rat was marked with a standard ring. Full thickness of the marked skin was then cut carefully.

The rats were randomly divided into three groups of 6 rats each. Group 1 rats were untreated (negative control). For Groups 2 and 3 rats, 5% and 10% *A. cordifolia* (stem) crude extract aqua-based cream (consisting of cetosteryl alcohol, sodium lauryl sulfate, white soft paraffin, phenoxyethanol and purified water) respectively, was applied topically once daily.

Wounds were traced on 1 mm^2 graph paper on the day of wounding and subsequently at an interval of 4 days, until day 16^{th}. This continues until healing was complete. Changes in the wound area were measured regularly. Significance in wound healing of the test groups is derived by comparing healed wound area on respective days with healed wound area of the control group. The results of wound closure are shown in Figures 17 and 18.

The percentage of wound closure was calculated with the formula below:

$$\text{Percentage of wound closure} = [1-(At/Ac)] \times 100 \qquad (4)$$

where At is wound area in treated rat in mm^2 and Ac is wound area in untreated rat in mm^2.

mark was left outside the capture image field but placed within the eye-piece field of view. The first image of the scratch was acquired.

The well plate was incubated in a tissue culture incubator at 37°C for 4 days. The plate was taken out of the incubator to be examined periodically and then returned to resume incubation. After the incubation, the well plate was placed under a phase-contrast microscope, the reference point was matched, the photographed region acquired was aligned and a second image was captured. The images acquired for each sample was analysed quantitatively by using computing software.

For each image, distances between the two side edges of the scratch was measured at certain intervals (µm) using Micro Imaging Software Standard cellSens. By comparing the images from time 0 to the last time point, the distance of each scratch closure based on the distances that are measured by the software was obtained. The images taken on day zero and on the fourth day for untreated scratch, and scratches treated with different concentrations of the *A. cordifolia* (stem) crude extract are shown in Figures 16A to H.

As can be seen in the images in Figure 16H, the scratch treated with the *A. cordifolia* (stem) crude extract of 15 mg/ml on Day 4 shows higher population of cells present at the scratch area compared to the scratches treated with the *A. cordifolia* (stem) crude extract of 3.75 mg/ml (Figure 16F), 7.5 mg/ml (Figure 16G) and untreated scratch (Figure 16E) on Day 4, respectively. The fibroblast cells on the edges of the scratch treated with the *A. cordifolia* (stem) crude extract of 15 mg/ml have migrated at a higher rate and proliferated to close the scratch.

The results show that the concentration of the *A. cordifolia* (stem) crude extract of this invention induces fibroblast cell migration and the *A. cordifolia* (stem) crude extract has proliferative capacity, which is essential in the treatment of wounds.

Table 14: Results of the wound closure area of Groups 1, 2 and 3 rats treated with different concentrations of *A. cordifolia* (stem) crude extract

	Group 1	Group 2	Group 3
	Negative control, Ac (mm^2)	5% *A. cordifolia* (stem) crude extract, At (mm^2)	10% *A. cordifolia* (stem) crude extract, At (mm^2)
Day 4th	8504	13351	13355
Day 8th	10683	15159	16112
Day 12th	10978	15395	16453
Day 16th	11994	15111	16503

Groups 2 and 3 rats shows the closure advanced by 1.23±5% and 1.32±6%, respectively, when compared to Group 1 rat. The result shows that the percentage of wound closure in the Group 3 rats treated with 10% of the *A. cordifolia* (stem) crude extract aqua-based cream is higher compared to other groups of rats. This confirms that the *A. cordifolia* (stem) crude extract of this invention has the ability to stimulate wound closure.

Conclusion

The *A. cordifolia* (stem) crude extract of this invention has been shown *in vitro* and *in vivo*, to be a plant extract that can singularly and effectively enhance the wound healing process.

The identification tests show that the *A. cordifolia* (stem) crude extract of this invention has different compounds and phytochemicals from those of *Passiflora edulis.* Due to the presence of different compounds and phytochemicals, the *A. cordifolia* (stem) crude extract of this invention demonstrates an increased efficacy in wound healing.

The antioxidant test shows that the *A. cordifolia* (stem) crude extract of this invention is capable of scavenging oxygen free radicals.

The *ex vivo* test using thoracic aortas of rats show growth of microvessels in the *A. cordifolia* (stem) crude extract treated aortic ring. This confirms that the *A. cordifolia* (stem) crude extract of this invention has pro-angiogenic activity which is essential to wound healing.

The *in vitro* test using mice fibroblast cells shows cell proliferation. This confirms that the *A. cordifolia* (stem) crude extract of this invention has the capability to promote cell proliferation which is essential to wound healing.

The scratch assay confirms that the *A. cordifolia* (stem) crude extract of this invention has the capability to increase wound closure rate and percentage.

Given the above, the inventors have demonstrated that the *A. cordifolia* (stem) crude extract of this invention is able to singularly and effectively address all phases of wound healing, i.e. inflammatory, proliferative and tissue modelling phases due to its antioxidant, cell proliferative and pro-angiogenic activities, respectively.

Importantly, the inventors have demonstrated that the *A. cordifolia* (stem) crude extract of this invention, even when administered alone, is particularly efficacious in the treatment of wounds.

More importantly, the *A. cordifolia* (stem) crude extract of this invention is a pure plant-based medicament, that can be safely administered to a human, orally and/or topically, in any acceptable form, for healing most types of wounds including non-healing chronic wounds such as a diabetic foot ulcer.

As will be readily apparent to those skilled in the art, the present invention may easily be produced in other specific forms without departing from its scope or essential characteristics. The present embodiments are, therefore, to be considered as merely illustrative and not restrictive, the scope of the invention being indicated by the claims rather than the foregoing description, and all changes which come within therefore intended to be embraced therein.

CLAIMS

1. Use of a plant extract derived from the stem of *Adenia cordifolia* in the manufacture of a medicament to be administered to a human for wound healing.

2. The use of claim 1 wherein said medicament is to be administered orally.

3. The use of claim 1 wherein said medicament is to be administered topically.

4. The use of claim 1 wherein said medicament is to be administered both orally and topically, concurrently.

5. The use of claims 2 and 4 wherein said medicament is to be administered in the form of pellets, tablets, capsules, powders, granules, suspensions or emulsions.

6. The use of claims 3 and 4 wherein said medicament is to be administered in the form of lotions, ointments, creams, powders, gels or transdermal patches.

7. The use according to any one of claims 2 to 6, wherein the plant extract is an ethanol extract.

8. The use according to any one of claims 2 to 7, wherein the plant extract comprises (E)-9-octadecenoic acid ethyl ester ($C_{20}H_{38}O_2$) and 9,12-octadecadienoic acid ethyl ester ($C_{20}H_{36}O_2$).