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RESEARCH ARTICLE

Phytochemical properties of a rare mangrove *Aegialitis rotundifolia* Roxb. leaf extract and its influence on human dermal fibroblast cell migration using wound scratch model

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ABSTRACT

Background: *Aegialitis rotundifolia* Roxb. is a small mangrove plant traditionally used for curing pain arising from sundry injuries. At present, there are no scientific evidences of its wound healing properties and few reports have claimed that reduction of pain could accelerate the wound healing process. **Aims and Objectives:** The present work deals with the qualitative and quantitative study of the phytoconstituents present in *A. rotundifolia* Roxb., ethanolic leaves extract and also evaluate its wound healing properties *in vitro*. **Materials and Methods:** The qualitative phytochemical analysis of the extract was performed using preliminary phytochemical tests whereas the quantitative determination of phytochemicals was evaluated spectroscopically using ultraviolet spectroscopy. The *in vitro* wound healing activity was determined on human dermal fibroblast (HDF) cells using the wound scratch assay. **Results:** The quantitative estimation of phytochemicals revealed that total phenolics was the most abundantly found phytochemical followed by a total flavonoid, total flavonol, total tannin, total alkaloid, total proanthocyanidin, and total saponin. In the *in vitro* wound healing assay, the sample was first examined for possible cytotoxicity on HDF cells where the results showed that it was slightly toxic at higher concentration (inhibit cell growth by 50%: 157.41 ± 3.4 µg/mL); thus, a concentration of 40 and 80 µg/mL was taken to carry out the scratch assay. The wound scratch assay revealed significant ($P < 0.01$) wound healing activity in a dose-dependent manner at 40 and 80 µg/mL concentrations. **Conclusion:** Thus, the extract proved effective in wound healing which may have been attributed by the presence of several bioactive phytochemicals.

KEY WORDS: *Aegialitis rotundifolia*; Mangrove; Phytochemical Analysis; Cytotoxicity; Wound Scratch Assay

INTRODUCTION

Mangroves have long been a source of huge astonishment along with abiding curiosity to the common men and of endless interest for the scientists.[1] The treatment of several diseases with the aid of plants and plant products is known as herbal medicine, which is considered as a part of the traditional and folk medicinal system. From the dawn of civilization, several ethnic groups have been using medicinal plants to combat several diseases as they were the only resource available for medication.[2]

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Wound can be defined as a disruption in the cellular and anatomical architecture and physiological function of tissue which includes skin, mucus membrane, deeply lying tissues or surface of internal organs ranging from incision,

laceration, abrasion, puncture, and closed wounds such as contusion, hematoma, and crush.[3] Impaired wound healing is considered a major problem which may be caused by excessive production of reactive oxygen species (ROS), microbial infection and uncontrolled inflammatory responses. On disruption of such tissues, intricate and complex processes are initiated to repair the damaged tissue which involves a complex cascade of cellular events resulting in reconstitution, resurfacing, and restoration of the tensile strength of the injured skin.[4] Wound healing is a complex biological process due to which several *in vitro* and *in vivo* methods are available. Among these models, the scratch assay has proven to be an important and valuable tool which provides first insights to how the natural products positively influence the regeneration of new tissues.[5,6]

Aegialitis rotundifolia Roxb. (*Plumbaginaceae*) is a small mangrove tree or shrub which usually grows up to a height of 2–3 m and is available in shorelines of the Andaman Sea and the Bay of Bengal and is endemic to the coastal parts of South Asia. In Orissa, it is locally known as Banrua.[7,8] This mangrove species is reported to produce one of the best quality honey.[9] Conventionally, the leaf is used in the treatment of sundry injuries accompanied by pain and inflammation and is locally utilized as an anti-acne agent.[10] Further, the leaf of the plant is pounded with oil to make a paste which acts as an antidote for insect bites.[11] According to the present literature, there have been very few scientific reports of pharmacological screening conducted such as analgesic, antipyretic,[11] *in vitro* antioxidant,[12] antimicrobial,[13,14] anti-inflammatory,[11,15] *in vitro* thrombolytic activity,[15] antibacterial,[15] and anticancer activity.[16] Recently, we have reported the presence of gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, rutin, coumarin, and quercetin by performing quantitative high-performance liquid chromatography (HPLC)_ analysis and an organosilicon compound, (-)-spiro[1-[(tert-Butyldimethylsiloxy)methyl]-3,5,8-trimethyl-bicyclo[4.3.0] non-2-en-5,7-diol-4,1'-cyclopropane] was detected in gas chromatography-mass spectrometry analysis as the most abundantly found compound.[8] However, there has been no scientific study conducted on the wound healing activity of the plant and also its phytochemical properties are also hugely unexplored. Therefore, the present study was designed to evaluate the qualitative and quantitative phytochemical analysis and also investigate the wound healing potential of *A. rotundifolia* using the scratch model on human dermal fibroblast (HDF) cell line.

MATERIALS AND METHODS

Chemicals and Reagents

Ethanol 99.9% used for extraction was procured from Changshu Hongsheng Fine Chemicals Co. Ltd., China. For *in vitro* wound healing studies, the required chemicals were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), and Thermo Fisher Scientific, Inc., (Waltham, MA, USA). All the solvents used were of high purity and HPLC grade. All other chemicals and reagents used in the whole study were of analytical grade.

Collection and Authentication of Plant Materials

The fresh leaves of *A. rotundifolia* Roxb. were collected from healthy fully grown plants from Bichitrapur Mangrove located in Kharibil, Orissa, India (21°34'54.0"N - 87°25'25.4"E). The plant materials were then authenticated from Botanical Survey of India, Central National Herbarium, Botanic Garden, Howrah, West Bengal, India, and were assigned with a Voucher no. CNH/Tech.II/2016/11a and specimen no. DG-01.

Preparation of Extracts

The collected plant materials were gently washed in tap water to remove dirt and were shade dried under room temperature (24 ± 2°C) for 3–4 weeks. After complete drying, the dried plant materials were pulverized by using a mechanical grinder followed by sieving to obtain a coarse powder. The powdered plant material was then extracted with ethanol (99.9%) using reflux technique. The crude extract solution obtained was filtered using Whatman No. 42 filter paper after which the excess solvents were evaporated by rotary vacuum evaporator (Evator, Media Instrument Mfg. Co., Mumbai, India) and concentrated on water bath to obtain *A. rotundifolia* Roxb., ethanolic leaves extract (ARELE). The crude ethanol extract obtained was stored at 4°C before analysis.

Qualitative and Quantitative Determination of Phytochemical Groups

For qualitative phytochemical analysis, preliminary phytochemical test was conducted on ARELE according to the standard procedures.[17-19]

For the quantitative estimation of phytochemical groups, the crude extract was screened for total phenolic content (TPC),[20] total flavonoid content,[21] total flavonol content,[22] total proanthocyanidin content,[23] total alkaloid content,[24] total saponin content,[25] and total tannin content[26] which were expressed as gallic acid equivalent (mg/g of extract), quercetin equivalent (mg/g of extract), quercetin equivalent (mg/g of extract), catechin equivalent (mg/g of extract), atropine equivalent (mg/g of extract), diosgenin equivalents (mg/g of extract), and tannic acid equivalent (mg/g of extract), respectively.

In vitro Wound Healing Activity Using Scratch Wound Healing Model

Cell culture and treatment

Cell lines HDF was procured from National Centre for Cell Science, Pune, and cultured in Ham's F-12 media supplemented with 10% inactivated fetal bovine serum (FBS),

penicillin (100 IU/mL), streptomycin (100 µg/mL), and amphotericin B (5 µg/mL) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% ethylenediaminetetraacetic acid, and 0.05% glucose in phosphate buffered saline [PBS]). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 well microtiter plates.

Preparation of Test Solution

The test drug was dissolved in dimethyl sulfoxide (DMSO) and volume was made up with Dulbecco's Modified Eagle's Medium-high Glucose (DMEM-HG) supplemented with 2% inactivated FBS to obtain a stock solution of 10 mg/mL concentration and sterilized by filtration. Serial two-fold dilutions were prepared from this for carrying out cytotoxic studies.

Assessment of *In vitro* Cytotoxicity Activity

The cytotoxic effect of the test extract on HDF cells was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay method.[27] The monolayer cell culture was trypsinized, and the cell count was adjusted to 1.0×10^5 cells/mL using DMEM-HG containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 mL of the diluted cell suspension was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off and then the monolayer was washed once with medium. Then, 100 µL of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37°C for 72 h in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µL of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100 µL of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values was generated from the dose-response curves for the cell line.

% Growth inhibition = 100 – (Mean OD of control group/ Mean OD of sample group) × 100

Scratch wound healing assay

The wound scratch assay was performed according to the procedure described by Liang *et al*.[28] HDF cells were cultured in 10% growth media (Ham's F-12) supplemented with FBS at 5% CO_2 and 37°C in a flask. The cells were reseeded in three 60 mm Petri dish at a density of 1.5×10^5 cells/mL. When the cells attained confluency (>70% cell density), serum-free growth media (DMEM-HG) were added and incubated overnight. After the incubation, a scratch of 0.8–1.0 mm width was created using a sterile 1 mL tip. The Petri plates were washed with PBS 2 times. The cells were treated with two safe concentration of the drug, keeping 1% DMSO as a control in three Petri dishes separately. The plates were observed at 0, 24, and 48 h. After 48 h incubation, the cells were washed with PBS and later fixed by adding 10% formaldehyde. Formaldehyde solution was discarded and 2 mL of 0.05% crystal violet was added and incubated for 5–10 min. The plates were observed under microscope and distance were measured using Motic microscope software at five different points for each intervals. Later, the cell migration was calculated by difference in intervals from 0 h to 48 h.

Statistical Analysis

All experiments were carried out in triplicate and the results are expressed as the average of three independent measurements (Mean ± Standard deviation). The data obtained in the studies were subjected to one-way of analysis of variance (ANOVA) for determining the significant difference. The intergroup significance was analyzed using Dunnett's *t*-test. $P < 0.01$ was considered to be significant. All the statistical analysis and data presentation were done using GraphPad InStat Version 3.06 (GraphPad Software, Inc. La Jolla, CA, USA) and Microsoft Excel 2013 standard (Microsoft Corp., Redmond, WA, USA).

RESULTS

Preliminary Phytochemical Screening

Preliminary phytochemical screening of ethanol extract from *A. rotundifolia* leaves revealed the presence of major phytochemical groups such as alkaloids, carbohydrates, tannins, steroids and sterols, triterpenoids, saponins, and flavonoids as shown in Table 1.

Quantitative Determination of Phytochemical Groups

The details of the phytochemical contents have been given in Figure 1. The TPC in the ethanol extract from *A. rotundifolia* leaves was 59.49 ± 0.82 mg GAE/g dry extract expressed from the gallic acid standard curve ($Y = 0.0146x + 0.164$, $r^2 = 0.9957$). Total flavonoid and flavonol content in the extract were found to be 51.54 ± 3.15 and 35.97 ± 1.70 mg QE/g dry extract, extrapolated from the standard quercetin curves: $Y = 0.0111x + 0.043$, $r^2 = 0.9943$ and $Y = 0.0131 x + 0.011$, $r^2 = 0.9982$, respectively. Total proanthocyanidin content was 9.16 ± 1.67 mg CE/g dry extract calculated from catechin standard curve: $Y = 0.0173x + 0.015$, $r^2 = 0.9986$. Total tannins in the extract were 23.62 ± 2.58 mg TAE/g dry extract obtained from tannic acid standard curve ($Y = 0.0059x + 0.1539$, $r^2 = 0.9926$). Total alkaloid and saponin were calculated from atropine standard curve ($Y = 0.0094x + 0.012$, $r^2 = 0.9982$) and diosgenin standard curve ($Y = 0.0014x + 0.0151$, $r^2 = 0.9993$) which was 12.54 ± 0.38 mg AE/g dry extract and

8.98 ± 0.54 mg DE/g dry extract, respectively. Thus, the phytochemical content in the extract was in the order: Total phenolic > total flavonoid > total flavonol > total tannin > total alkaloid > total proanthocyanidin > total saponin.

In vitro Wound Healing Activity Using Scratch Wound Healing Model

In vitro cytotoxicity activity

The inhibitory effects of ARELE on the cell viability of the HDF cell line were investigated using MTT assay, and the results are shown in Figures 2 and 3. To this purpose, the HDF cell lines were treated with various concentrations of ARELE (7.2–1000 µg/mL) where the results revealed that ARELE showed the cytotoxic effect on HDF cells at a high concentration level with a CTC_{50} value of 157.41 ± 3.4 µg/mL. Thus, at low concentration, ARELE can be considered safe for carrying out further studies. Based on the above cytotoxic study, two safe doses of ARELE (40 µg/mL and 80 µg/mL) were considered for wound scratch assay.

Scratch wound healing assay

The results of the wound scratch assay are shown in Figures 4 and 5. Scratch was created to mimic wound and the capacity of these HDF cells to migrate and cover the scratch was captured using a camera attached to a light microscope and the distance covered was measured and analyzed quantitatively at a time interval of 0, 24, and 48 h, after the creating the scratch. The results revealed that ARELE exhibited significant (P <0.01) efficacy in wound healing when compared with control by offering 352.88 ± 3.92 µM and 238.01 ± 2.81 µM migration of HDF cells at 80 and 40 µg/mL, respectively, after 48 h. Reference standard cipladine also exhibited significant (P < 0.01) wound healing after 48 h when compared with control at 5 µg/mL where the distance covered was 447.46 ± 2.40 µM. At 24 h the distance covered by HDF cells after treatment with ARELE 80 µg/mL, ARELE 40 µg/mL, and reference standard cipladine was 293.48 ± 3.84, 145.22 ± 1.41, and 356.66 ± 1.49 µM, respectively. Thus, it can be inferred that ARELE at low concentration showed considerably high wound healing efficacy in a dose-dependent manner.

Table 1: Preliminary phytochemical test of the ethanol extract of *A. rotundifolia* Roxb. leaves

Phytochemicals	Tests performed	Inference
Alkaloids	Mayer's test	+
	Dragendorff's test	+
	Wagner's test	+
	Hager's test	+
Carbohydrates	Molisch's test	+
	Fehling's test	+
	Benedict's test	+
Proteins and amino acids	Biuret test	–
	Ninhydrin test	–
	Xanthoproteic test	–
	Millon's test	–
Tannins	Ferric chloride test	+
Steroids and sterols	Lieberman–Burchard test	+
	Salkowski's test	+
Triterpenoids	Sulfuric acid test	+
Cardiac glycoside	Keller-killiani test	–
Saponins	Foam test	+
Flavonoids	Shinoda test	+
	Ferric chloride test	+
	Lead acetate test	+
	Zn dust test	+

A. rotundifolia: Aegialitis rotundifolia
(-) Absent, (+) Present

DISCUSSION

The present work deals with the qualitative and quantitative phytochemical analysis and evaluation of *in vitro* wound healing activity on HDF cells, thus providing for the 1st time the scientific aspects of the plant's potential as a wound healing agent. The preliminary phytochemical test of ARELE revealed the presence of alkaloids, carbohydrates, tannins, steroids

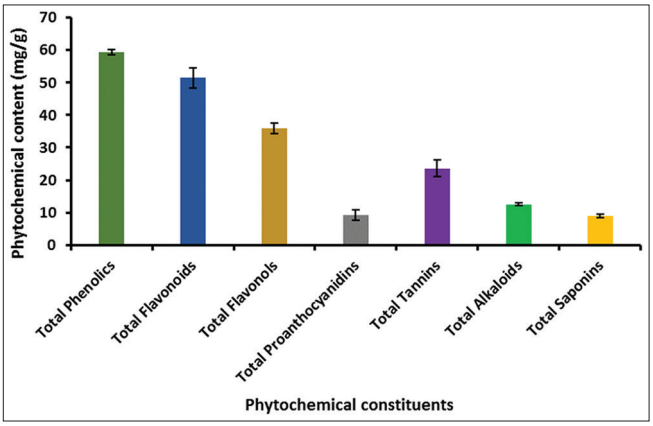

Figure 1: Phytochemical constituents present in *Aegialitis rotundifolia* ethanolic leaves extract determined quantitatively

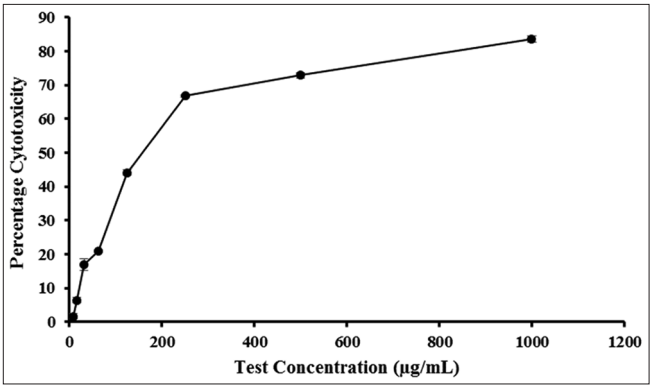

Figure 2: Cytotoxic effect of ethanol extract from *Aegialitis rotundifolia* leaves on human dermal fibroblast cell line

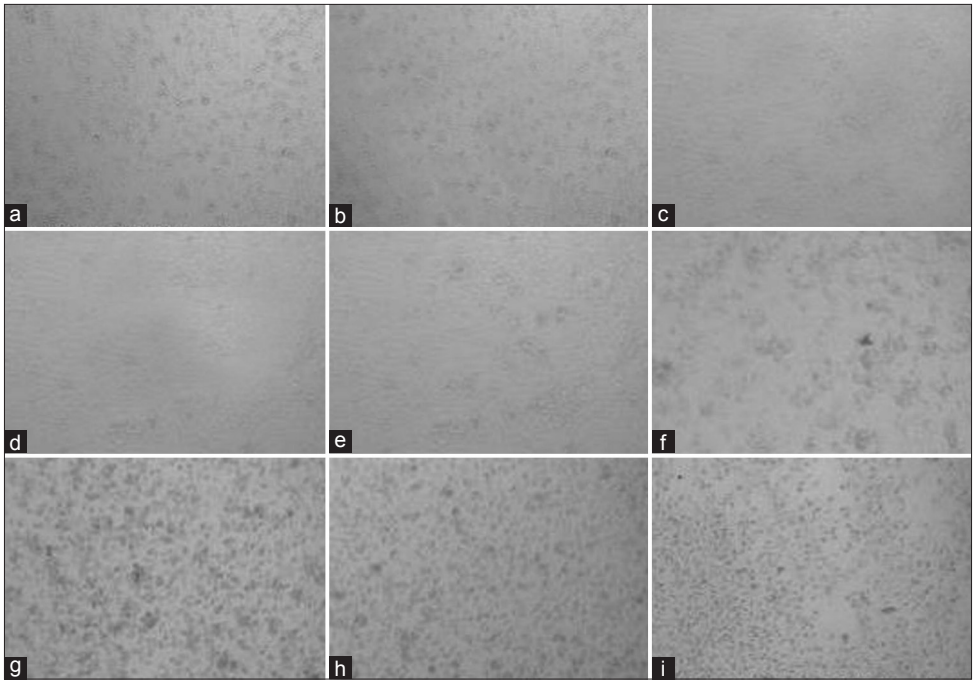

Figure 3: Microscopic images showing cytotoxic activity of *A. rotundifolia* Roxb., ethanolic leaves extract (ARELE) on human dermal fibroblast cell line. (a) Normal control; (b) ARELE (7.2 µg/mL); (c) ARELE (15.6 µg/mL); (d) ARELE (31.2 µg/mL); (e) ARELE (62.5 µg/mL); (f) ARELE (125 µg/mL); (g) ARELE (250 µg/mL); (h) ARELE (500 µg/mL); (i) ARELE (1000 µg/mL)

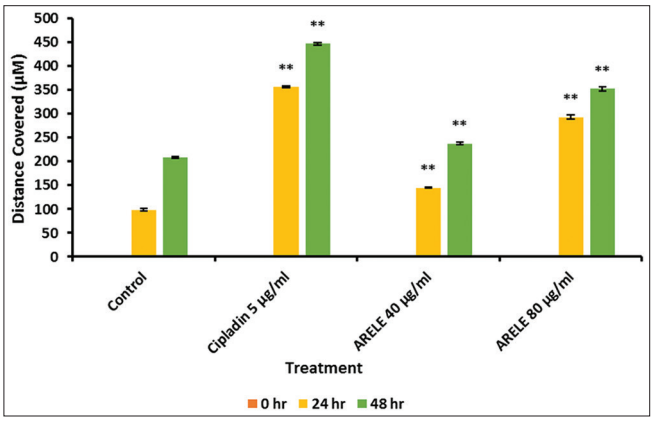

Figure 4: Effect of *A. rotundifolia* Roxb. ethanolic leaves extract on human dermal fibroblast cell migration in wound scratch assay studied at 0 h, 24 h, and 48 h. The values are expressed as mean ± standard deviation of triplicate determinations. Statistical analysis done by one-way ANOVA followed by Dunnett's t-test. **P < 0.01 compared to control group

and sterols, triterpenoids, saponins, and flavonoids which may lead to their quantitative estimation and also identifying the source of pharmacologically active phytoconstituents.[17] Quantitative phytochemical content in ARELE was analyzed and the result was found to be in the following order: Total phenolic > total flavonoid > total flavonol > total tannin > total alkaloid > total proanthocyanidin > total saponin. The cytotoxic activity of ARELE on HDF cells was performed to find out any possible toxicity of the extract on the HDF cells, and the result revealed that ARELE was cytotoxic at a high concentration level with a CTC_{50} value of 157.41 ± 3.4 µg/mL. Thus, the low concentration of ARELE was considered safe for carrying out the wound scratch assay. The wound scratch assay was performed after the cytotoxicity activity, and the results showed that ARELE was able to accelerate the HDF cell migration toward the scratch or mimicked wound thus showing significant ($P < 0.01$) wound healing activity *in vitro* in a dose-dependent manner.

Plant kingdom harbors an almost inexhaustible source of active phytoconstituents responsible for the treatment of various diseases.[29] According to traditional knowledge, this mangrove species is used as a pain relieving agent by various local healers in mangrove areas.[8] In a report published by Mudge and Orsted,[30] pain is often associated with wounds which may arise due to damage of tissues (nociceptive pain) or abnormal functioning of the nervous system (neuropathic pain). According to McGuire *et al*.,[31] pain is also responsible for delayed wound healing process as they are known to dysregulate neuroendocrine and immune function which are known to play a key role in wound repair mechanism. Thus, herbal preparations with potent pain-relieving activity can be potentially employed for accelerating wound healing process. According to the current literature, there is no investigation done on the wound healing properties of this mangrove plant; however, its strong ameliorating effect on pain and inflammation is scientifically reported previously by Raju *et al*.[11]

Phenolic compounds are considered very important phytochemical group which are responsible for protection of tissue membranes and proteins against harmful free radicals.[32,33] Our study showed that high phenolic content was present in the test extract which was slightly more

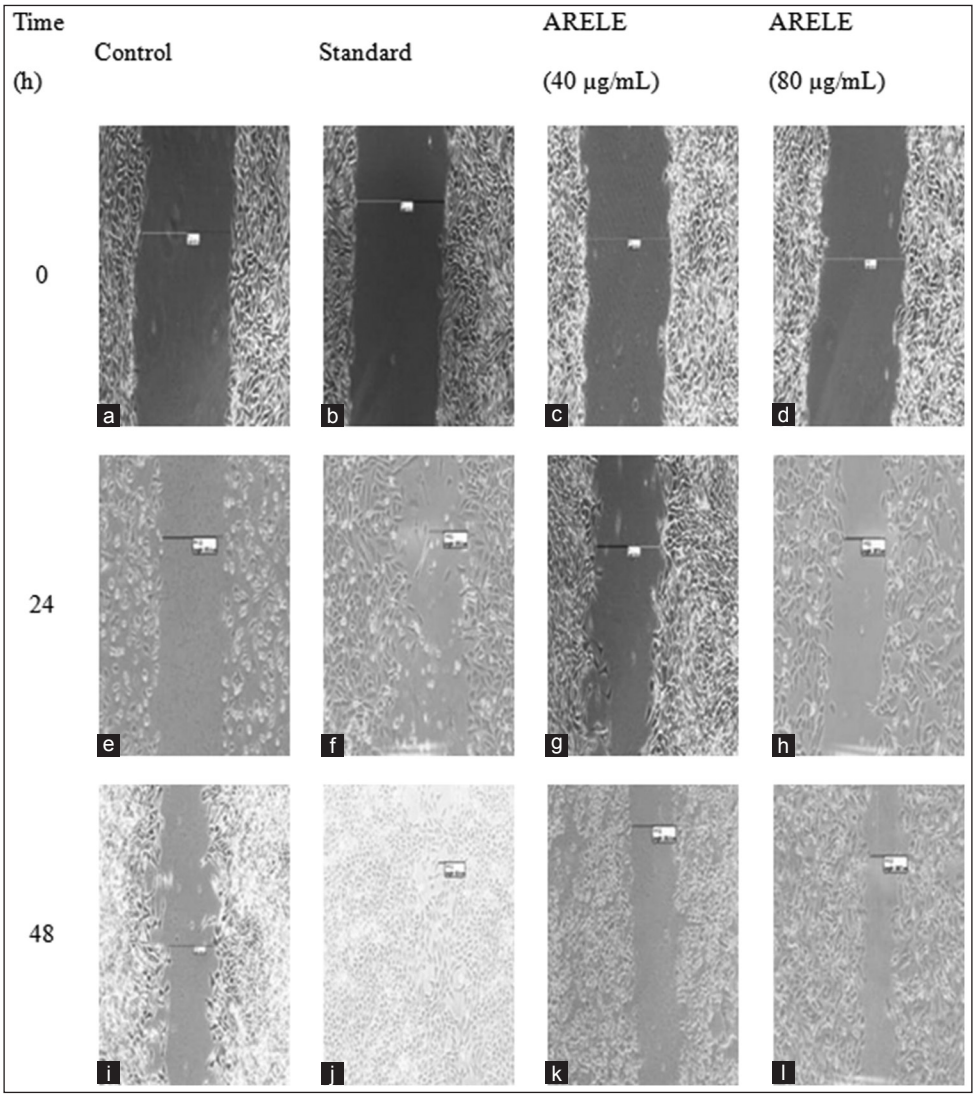

Figure 5: Microscopic images of human dermal fibroblast cells migration after scratch

than the previous finding of TPC in *A. rotundifolia* leaves studied by Sett *et al*.[34] Flavonoids are hydroxylated phenolics possessing a benzo-γ-pyrone structure which naturally occur in plants and have a positive effect on human health.[35] Flavonoids are also reported to possess strong wound healing properties as revealed by Lodhi and Singhai, 2013.[36] The high amount of flavonoid content was found in the test extract. Flavonols are a class of flavonoids that possess the 3-hydroxyflavone backbone.[37] Our study showed considerable flavonol content in the test extract. Proanthocyanidins are polyphenolic bioflavonoids which have a protective effect by eliminating hydroxyl radicals.[38] Our findings revealed low proanthocyanidins content in ARELE. Tannins are phenolic biomolecules which have protective properties against various diseases.[39] The tannin content in the extract was found to be moderately high. Alkaloids are naturally occurring compounds which are mostly composed of basic nitrogen atoms and have diverse and important physiological effects on humans and animals. They are also said to possess several therapeutic properties such as analgesic, antimalarial, antiseptic, bactericidal, and anti-inflammatory.[40] In our investigation, alkaloid content was comparatively high with respect to total saponin and proanthocyanidins. Saponins are glucosides with foaming characteristics and are mostly associated with cell growth and inhibition in humans and producing several healing effects including wound healing properties of saponins which were previously studied by Rajput *et al*.[41] The saponin content in the test extract was found to be low compared to other phytochemical groups.

Healing of wounds is accompanied by inflammation and formation of new tissues. At the beginning of new tissue formation, it is observed that migration of keratinocytes/fibroblast cells of the injured epidermis and hair follicles takes place followed by cell proliferation at the wound site. This process is described as re-epithelization. Finally, the re-differentiation of keratinocytes/fibroblast cells takes place to restore the barrier function. Thus, bioactive compounds which are able to stimulate fibroblast growth and migration may be able to improve or accelerate wound healing.[28,42] In our present study, the wound healing capacity of ARELE was

tested in HDF cell lines using scratch model. The scratch assay is a method of determining wound healing *in vitro* and is based on the observation that on creation of scratch or artificial gap on a confluent cell monolayer, the cells on the edge of the newly created scratch will travel toward the opening to cover the created scratch until new cell-cell contacts are formed again. This assay is considered economical and straightforward for studying cell migration *in vitro*.[28] The results of our study revealed a significant ($P < 0.01$) wound healing activity *in vitro* in a dose-dependent manner. In our previous work,[8] polyphenolic compounds such as gallic acid, chlorogenic acid, rutin, and quercetin were detected in *A. rotundifolia* leaves all of which possesses potent wound healing activity.[43-46] The presence of these polyphenolics in the extract guarantees a potential activity of curbing ROS and promoting the process of wound healing since a high amount of ROS is produced at the injured site.[47]

The plant is traditionally used to cure pain caused by sundry injuries and is also applied topically thus this work will provide scientific information for the 1st time about the plant's possible cytotoxic effects on HDF cells and also its wound healing properties; thus, validating its safety on HDF cells and traditional use. This work will further give a more in-depth information about the phytochemical content as this mangrove plant is considered rare and its phytochemical properties have not been explored much before.

CONCLUSION

In vitro wound healing assay was performed on normal HDF cells using scratch assay which revealed significant activity in a dose-dependent manner. The wound healing potential of ARELE may be attributed due to the presence of several phytoconstituents which has been estimated both qualitatively and quantitatively. This work gives the first report of the wound healing potential of *A. rotundifolia* leaves and also gives a much in-depth information of its phytochemical properties. This study can also be taken as a benchmark for further investigation to identify the phytoconstituents which are responsible for the wound healing activities and also study their mechanism of actions.

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