

## **SYZYGIUM AROMATICUM (L.) EXTRACTS STIMULATE THE CELL PROLIFERATION IN AN *IN VITRO* SCRATCH ASSAY**

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### **ABSTRACT**

Wounds have always been a serious hazard to the public health around the world. Currently available treatments are limited due to their side effects and high cost. Plants are being used as a good source of medicines due to their safety, and ease of access. The objective of the study was to examine the potential cell proliferation and migration activities of *Syzygium aromaticum* extracts to understand the wound healing property of this plant. Methanol-, ethanol- and acetone-based extracts were prepared from *S. aromaticum* flower buds and tested in a scratch assay using a human retinal pigment epithelial (RPE) cell line. Polyphenolic contents in the extracts of *S. aromaticum* responsible for wound healing potential were identified using high performance liquid chromatography (HPLC). The study revealed that the majority of examined extracts stimulated cell proliferation of RPE cells. The activities were highest when the extracts were prepared using acetone as solvent at 0.05 mg/mL concentration. Acetone- and ethanol-based extracts yielded the best results at 0.05 mg/mL whereas methanol-based extract produced the highest result at 0.5 mg/mL. Thus, the present study provides better understanding of the traditional use of *S. aromaticum* for the treatment of wounds. However, further detailed studies are needed to optimize the concentration of different compounds of *S. aromaticum* for wound healing treatment.

**Key words:** Bioactive compound; Cell scratch assay; Polyphenol; Traditional medicine

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### **INTRODUCTION**

Wound has been a devastating problem since prehistoric times; about 20 million cases of wounds are being reported annually (Koehler *et al.*, 2018). The international market for wound care and associated problems is anticipated to increase to 22 billion dollars by 2024 (Sen, 2019). Wound healing is a programmed process containing 4 distinctive but somehow interrelated stages: hemostasis, inflammation, proliferation, and remodeling. The delay or any obstacle in the normal processing of these stages leads to chronic, non-healing wounds. It impose a substantial burden to the patients as well as to the medicare system (Han and Ceiley, 2017). Treatment of wounds has become even more challenging due to underlying conditions ranging from stress to malnutrition to metabolic syndrome (Sen, 2019).

Plant extracts, their ointments and other preparations are progressively being studied for potential health benefits and treat some chronic diseases e.g., cancer, hyperlipidemia, diabetes, and wounds (Schmidt *et al.*, 2009; Buranrat *et al.*, 2020). Approximately 80% of the people worldwide utilize herbal treatments for primary health care. The interest in using plants and herbs in modern medicine has increased during the past few decades (Blumenthal *et al.*, 2006).

*Syzygium aromaticum* has traditionally been used to treat inflammation, toothache and to stimulate the healing process of acute as well as chronic wounds. Enhanced collagen synthesis has been reported in dermal fibroblast cells using *S. aromaticum* oil. Additionally, polyphenols i.e., gallic acid and ellagic acid present in the extract of *S. aromaticum* were studied to enhance wound healing (Yang *et al.*, 2016). Bioactive phenolic compounds present in *S. aromaticum* have also been reported to be responsible for antioxidant, antidiabetic, anticarcinogenic, antifungal, antibacterial, antiviral, and mosquito repellent activities (Mittal *et al.*, 2014).

Cell proliferation activities of different plant extracts and bioactive compounds can be determined using various *in vivo*, *ex vivo* and *in vitro* models. *In vitro* methods are usually simple, less time consuming, economical and involve less ethical deliberations compared to animal models (Gottrup *et al.*, 2000). Scratch assay is the simplest and most efficient procedure to investigate *in vitro* cell proliferation studies (van Horssen *et al.*, 2006). The main steps involved in a scratch assay are creating scratch on a layer of cells, capturing pictures at the start and after proper intervals to evaluate the movement of cells, and evaluation of the images to assess the degree of wound healing (Liang *et al.*, 2007; Zubair *et al.*, 2012). The study was designed to

explore the wound healing activity of *S. aromaticum* in an *in vitro* cell scratch assay and to quantify the bioactive phenolics for wound healing activity of *S. aromaticum*.

## MATERIALS AND METHODS

**Sample collection, identification and extraction:** *Syzygium aromaticum* flower buds were collected from a local market in March 2019. Flower buds were identified by Dr. Muhammad Azeem, Department of Botany, Government College University Faisalabad, Pakistan. Voucher seed specimen, number SA-17-1, is kept at the Department of Bioinformatics and Biotechnology, Government College University Faisalabad, Pakistan. The samples were washed, weighed, and placed in spontaneous convection oven (Memmert, Germany, Schutzart DIN EN 60529 – IP 20) at 30°C for drying. These samples were crushed with a mill and extracted in 50% ethanol, 50% methanol and 50% acetone (dissolving 1 g of *S. aromaticum* powder in 10 mL solvent). Solutions were sieved using Whatman filter paper No. 1. The solvents were vaporized by rotatory evaporator and crude extracts were dissolved in phosphate buffer saline (PBS) 1 x (pH 7.4) for further analysis, which were performed between May-September 2019, at Health Biotechnology Lab. Government College University, Faisalabad, Pakistan (Zubair *et al.*, 2012b).

**Cell line culture:** Retinal pigmented epithelial (RPE) cells were provided by Dr. Muhammad Farooq (Department of Bioinformatics and Biotechnology, Government College University, Faisalabad, Pakistan). The cells were sustained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). These cells were placed in a CO<sub>2</sub> incubator at optimum conditions i.e., 37°C and 5% CO<sub>2</sub> for 24 hours. Once the cells were 80–100% confluent and were actively proliferating 2–5 mL of trypsin was added to detach the cells from the surface of the flask. Cells were diluted at a concentration of 2×10<sup>4</sup> cells/mL containing 10 mL of DMEM for further reattachment and division (Zubair *et al.*, 2012b).

**Scratch assay:** The proliferation ability of RPE cell line was evaluated by scratch assay with slight modifications from (Liang *et al.*, 2007). For this purpose, cells were cultured in a 12 well plate (used for cell culture) at 2×10<sup>4</sup> cells/mL, containing same medium and placed at conditions as described above. A straight scratch of approximately 1 mm width was generated by using a sterilized pipette tip (200 µL). Cells were washed using PBS. For experimental purpose PDGF (platelet derived growth factor- 5 ng/mL final concentration) and PBS were used as positive control and negative control respectively. Extracts were tested at four different concentrations (5, 0.5, 0.05, and 0.005 mg/mL).

Digital Pictures of the scratch on cell monolayer were captured with Meiji Techno TC5200- invert phase microscope at 0, 10 and 16 hours after creation of the scratch. Two pictures of each well of cell culture plate were taken at 40x magnification. These pictures were then assessed at 1712×1368 pixels with Image J software version 1.440. Scratch width at specific time intervals was measured and then compared to the scratch width at 0 hours after the creation of scratch. For each time interval 100 measurements were taken, and these measurements thus helped us to describe cell migration and proliferation.

**HPLC analysis:** Polyphenolic contents present in the extracts of *S. aromaticum* were determined using HPLC diode array detection system. Polyphenols were separated using a C-18 column of size 25 cm x 4.6 mm, 5 µm at 25°C from 10 µL of extract. Compounds were eluted isostatically at 1 mL/min (flow rate). The mobile phase consists of solution A (5% acetonitrile with 1% acetic acid in dH<sub>2</sub>O) and solution B (5% methanol with 95% acetonitrile). Filtration of solution A was done through 2.5 µm isopore membrane filter before use. The gradient for the mobile phases was, solution A: B, 82% and 18% for 0–12 min; 79% and 21% for 12–16 min; 80% and 20% for 16–18 min; 82% and 18% for 18–21 min and then 82% and 18% for 25 min. Polyphenols were quantified at 280 nm. Measurements were done in constant chromatographic conditions (Zubair *et al.*, 2011, 2012a).

**Statistical analysis:** Data from measurements of pictures of acetone-, ethanol- and methanol-based extracts of *S. aromaticum* tested at 5, 0.5, 0.05 and 0.005 mg/mL, negative and positive control was arranged in the Microsoft Office Excel 2010 for windows. Wound contraction percentage of each observation taken from Image J 1.440 was calculated in the Microsoft Office Excel 2010. Data sets generated from Microsoft excel were then sent to GraphPad Prism 6. Data from the experiments was analyzed using One-way Analysis of variance (ANOVA) and Tukey's post hoc test ( $p < 0.05$ ) to disclose differences among treatment means as described previously (Zubair *et al.*, 2012b).

## RESULTS

The potential cell proliferation ability of *S. aromaticum* was evaluated on RPE cells. The results revealed that cells treated with *S. aromaticum* extracts proliferate over scratch faster than the cells treated with PBS. The complete closure of scratch was obtained after 24 hours. However, the most reliable results were obtained after 16 hours of incubation, where clear differences among the extracts were observed (Fig. 1).

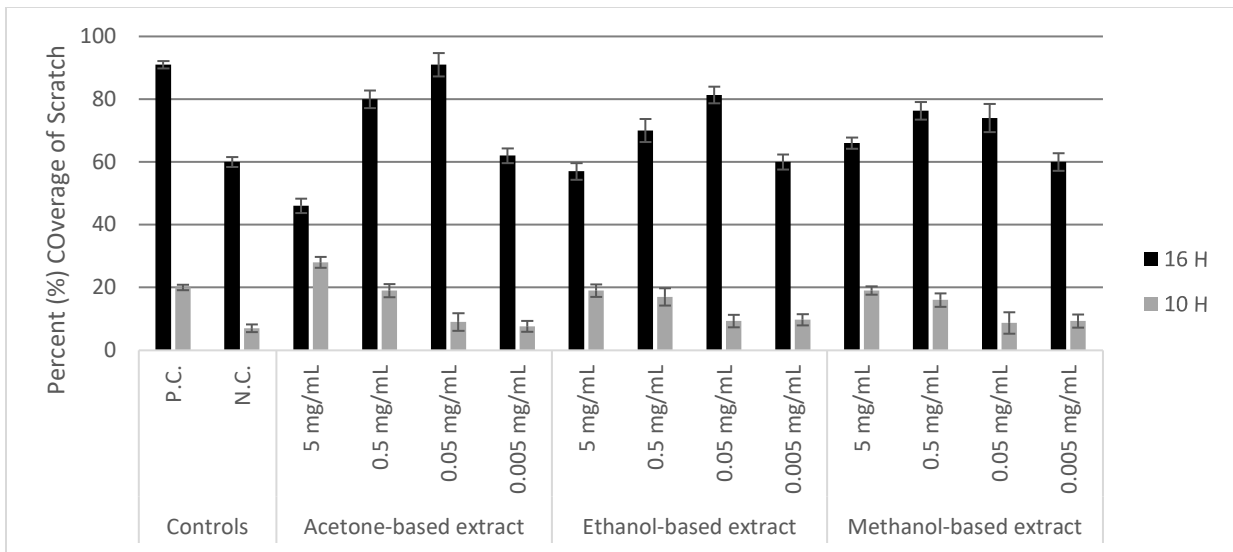


Figure 1. Effects of *Syzygium aromaticum* extracts on cell proliferation/migration in a scratch assay. Bars display the mean  $\pm$  S.E.M (on the basis of 3 repeats of every treatment group and 100 measurements for every repeat).

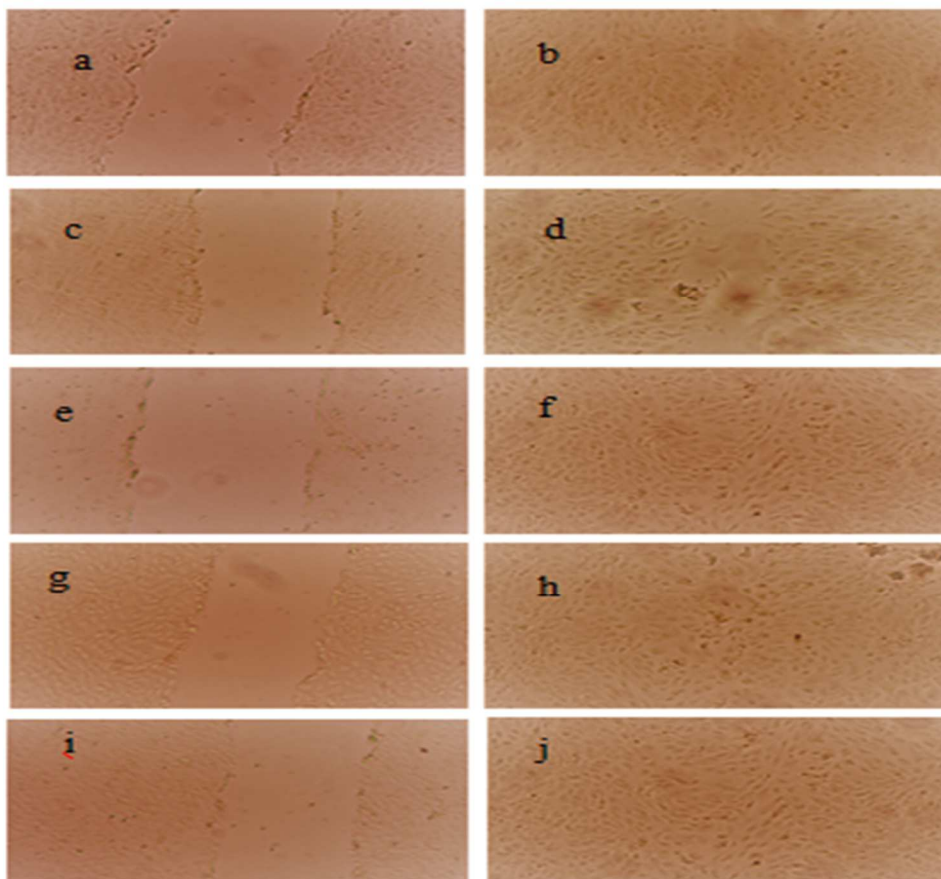


Figure 2. Wound healing activity of *Syzygium aromaticum* extracts at 0 and 16 hours of incubation after the wound creation. a and b are treatment with positive control at 0 and 16 hours respectively, c-d represents treatment with negative control, e-f are treatment with acetone-based extracts at 0.05 mg/mL. g-h represents treatment with ethanol-based extract at a concentration of 0.05 mg/mL. i-j represents treatment with ethanol-based extract at a concentration of 0.5 mg/mL.

Analysis of variance performed for each time point, confirmed the substantial differences ( $p < 0.05$ ) among the extracts. Tukey's post hoc test displayed substantial differences between extracts prepared by using different solvents. (Table 1 and 2). The percent wound coverage by the extracts of the *S. aromaticum*, the negative control and the positive control confirms the healing activity of all the extracts (Fig. 2). Under optimized conditions the proliferation ability of RPE was highest at 0.05 mg/mL for extracts of *S. aromaticum* prepared by using ethanol and acetone as solvent, but at 0.5 mg/mL using methanol as solvent. Overall, acetone-based extract had the highest wound healing activity followed by ethanol and methanol-based extract (Fig. 2).

The HPLC analysis of *S. aromaticum* confirmed the presence of three major polyphenolic compounds i.e., gallic acid, eugenol, and ellagic acid. Among these identified polyphenols eugenol was present in highest concentrations in all extracts followed by gallic acid and ellagic acid. Concentrations of polyphenols in extracts, expressed as  $\mu\text{g/g}$  of dry weight have been given in Table 3. Considerable differences were found in concentrations of gallic acid and eugenol using different solvents; however, the concentration of ellagic acid was almost similar in all the extracts prepared by using different solvents. Acetone-based extract contains the maximum concentrations of the compounds than the extract prepared using ethanol and methanol.

**Table 1. Assessment of cell proliferation activity of *Syzygium aromaticum* extracts using analysis of variance.**

Interval (h)	Variable	DF	MS	F-value	P-value
10	Extracts	13	868.772	330	0.000
16	Extracts	13	1311.234	452	0.000

**Table 2. Tukey's post hoc test for identifying statistical differences in pixels among the wound healing activity of *Syzygium aromaticum* extracts in a scratch assay.**

Extract	Conc. (mg/mL)	10h	16h
Acetone	5	29.16 a	50.81 e
Ethanol	5	17.27 c	55.51 e
Methanol	5	18.23 c	68.51 d
Acetone	0.5	18.47 c	82.01 b
Ethanol	0.5	14.97 d	72.01 cd
Methanol	0.5	13.56 d	78.01 c
Acetone	0.05	6.57 e	93.18 a
Ethanol	0.05	7.10 e	84.27 b
Methanol	0.05	6.34 e	76.18 c
Acetone	0.005	6.18 e	64.26 d
Ethanol	0.005	7.34 e	61.96 d
Methanol	0.005	6.99 e	62.97 d
PBS (Negative)		6.18 e	62.39 d
<sup>a</sup> PDGF (Positive)		22.36 b	92.66 a

Significant differences ( $p < 0.05$ ) between different extracts displayed by alphabets (a–e) in same column.

**Table 3. Tukey's post hoc test for statistical significance among the concentration of polyphenols ( $\mu\text{g/g}$  of dry weight) extracted from *Syzygium aromaticum* bud using different solvents.**

Polyphenolic compound	Acetone-based extract	Ethanol-based extract	Methanol-based extract
Gallic acid	107 a	90 b	85 b
Ellagic acid	96 a	93 a	87 a
Eugenol	255 a	220 b	198 c

Significant differences ( $p < 0.05$ ) between different extracts displayed by alphabets (a–e) in same row.

## DISCUSSION

Cell proliferation is a dynamic phase in wound healing. *In vitro* cell proliferation of different cell lines using several plant species have frequently been studied

via scratch assay (Schmidt *et al.*, 2009; Sevimli-Gür *et al.*, 2011; Ranzato *et al.*, 2011). Optimum period for evaluation of scratch width after the creation of scratch was 12–72 hours depending on the plant extracts, cell types and width of scratch (Liang *et al.*, 2007; Zubair *et al.*, 2012). While, in present research scratch area was

completely covered within 24 hours and consistent results were observed after 16 hours. The present study suggests that optimum time to evaluate the proliferation ability of extracts of *S. aromaticum* on RPE cell line was 0–16 hours under optimized conditions (5% CO<sub>2</sub> and 37°C). Our study demonstrates a substantial stimulation in cell proliferation using the *S. aromaticum* extracts. The results of the scratch assay indicate that sub/super optimal concentrations of *S. aromaticum* extract may have anti-proliferative effects. Previous studies using jojoba liquid wax and ellagic acid have also shown similar results (Ranzato *et al.*, 2011; Primarizky *et al.*, 2017).

The study is in line with the fact that plants have traditionally been used to induce the healing process of wounds. The bioactive compounds responsible for the cell proliferation activities of *S. aromaticum* were previously not known. Among the major identified compounds revealed in extracts gallic acid and ellagic acid have previously been found to have positive effects on cell proliferation (Inam *et al.*, 2014; Yang *et al.*, 2016). Whereas, based on the results of our investigations, it appears that the *S. aromaticum* extracts containing polyphenols (0.480 µg/mL of ellagic acid, 1.275 µg/mL of eugenol and 0.535 µg/mL of gallic acid) may have promoted cell proliferation. However, further studies are required to optimize the concentration of specific polyphenols/synergistic effects of above-mentioned compounds.

The polyphenols i.e., quercetin, gallic acid, quercetin glucoside, eugenol and ellagic acid have been identified in *S. aromaticum* extracts previously. Eugenol has been reported in highest concentration in almost all the previous studies using ethanol or methanol as solvent. The concentration of eugenol and other major compounds have however been reported to be different using different solvents, drying method/temperature, harvesting method and post-harvest handling (Inam *et al.*, 2014; Higashi, 2015). The use of various solvents for extracting polyphenols have identified acetone, methanol, and ethanol as most effective solvents. Among them acetone has been reported to produce the best results for extraction of mango seeds (Dorta *et al.*, 2012) elderberry and grapes (Vatai *et al.*, 2009). In the present study, extracts prepared using acetone contain the highest amount of the identified polyphenolic compounds. These results were consistent with the literature.

**Conclusion:** *Syzygium aromaticum* extracts stimulated the proliferation ability of cells in an *in vitro* scratch assay using RPE cell line. Among tested concentrations, 0.05 mg/mL of acetone- and ethanol-based extracts was found more effective than higher concentrations (5 and 0.5 mg/mL) and lower concentration (0.005 mg/mL). While in case of methanol-based extract 0.5 mg/mL of *S. aromaticum* significantly promoted the cell proliferation compared to all other tested concentrations (5, 0.05, and

0.005 mg/mL). The acetone-based extracts were found to possess highest concentration of major polyphenols among the solvents used, and *in vitro* cell proliferation/migration abilities of *S. aromaticum* were highest when the extracts were made using acetone as solvent at 0.05 mg/mL. The concentration represents 5.35 ng of gallic acid, 4.3 ng of ellagic acid and 11.25 ng of eugenol per mL of the cell culture medium. However, further research is required for the identification of specific polyphenols having wound healing potential and to optimize the concentration of these compounds.

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