Journal of Medicinal Plants and By-Products

Original Article

Exploring the Therapeutic Potential of Malaysian *Stevia rebaudiana* Bertoni leaves essential oil: A Comprehensive Study on Cell Viability and Biological Effects for Topical Applications

Nur Izzati Mohamad Zen[1], Uswatun Hasanah Zaidan[1,2*], Siti Salwa Abd. Gani[1,3], Suhaili Shamsi[2], Masriana Hassan[4] and Nazrim Marikkar[5]

[1] *Halal Products Research Institute, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

[2] *Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

[3] *Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

[4] *Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

[5] *National Institute of Fundamental Studies Hanthana Road, 20000 Kandy, Sri Lanka*

[*]Corresponding Author: Email Address: uswatun@upm.edu.my; Tel.: +603-97697620 Fax.: +603-97697950

Article History: Received: 08 March 2023/Accepted in revised form: 23 December 2023

ABSTRACT

Background: The abundance of plant resources in Malaysia has gained the attention of many researchers to explore more pharmacological effects of plant's essential oil (EO). EO from *Stevia rebaudiana* Bertoni (SrB-EO) leaves consists of a rich blend of a variety of volatile molecules and bioactive compounds which currently attract the attention of a lot of researchers due to its high potential to play a role in the therapeutic activity. **Methods:** In the present study, SrB-EO used was extracted using a Soxhlet extractor in absolute ethanol. The cell viability on SrB-EO has been investigated using Hs27 skin fibroblast cells against two biological assessments; anti-inflammatory and antimicrobial assays. **Results:** It shows that 500 µg/mL was the highest concentration of SrB-EO which is considered non-toxic (82.08% cell viability) and demonstrates a primary significant cell proliferative effect toward cells. The maximum inhibition percentage of nitrite of SrB-EO was 33.12%, which showed a good anti-inflammatory property and SrB-EO showed an antimicrobial effect toward Gram-positive bacteria; *Bacillus subtilis* B29 and *Staphylococcus aureus* ATCC 43300. The minimal inhibition concentration (MIC) of *B. subtilis* and *S. aureus* were 6.25 mg/mL and 3.13 mg/mL of SrB-EO respectively, and the minimal bactericidal concentration (MBC) of *B. subtilis* and *S. aureus* were 12.50 mg/mL and 10.42 mg/mL of SrB-EO, respectively. **Conclusion:** This study concluded that a low concentration of SrB-EO is non-toxic toward human skin cells and these EOs possess satisfactory anti-inflammatory and antibacterial properties. Thus, SrB-EO can be used as an alternative therapeutic agent for topical application in the medicinal field.

Keywords: Essential oil, *Stevia rebaudiana* Bertoni, Cytotoxicity, Anti-inflammatory, Antimicrobial activity

INTRODUCTION

Stevia rebaudiana Bertoni is a herb from *Asteraceae (Compositae)* family which is also known as sweet herb, sweet leaf, honey yerba, honey leaf, and candy leaf. Stevia grows in sandy soil near streams and north-eastern native to Paraguay. It was first found in Japan in 1968. *Stevia rebaudiana* species is the sweetest among other 18 species that have sweetening properties, from 150-300 species of stevia and has been described as the 'sweetness of the future' due to its calorie-free natural sweetness taste. The leaves of stevia are used as natural sweetener with health benefits like other herbs. It has been reported to have numerous medicinal properties, for instance antiviral, antifungal, antimicrobial, anti-hyperglycaemic, antihypertensive, and antitumour effects [1, 2]. However, these biological properties come from the secondary metabolites of aromatic EO [3]. EO are volatile, transparent, and rarely coloured liquid, lipid soluble, and soluble organic solvent with generally lower density than water [4]. Mostly EO from plants are used in food, drugs, and perfumery purposes. EO act as the 'chemical weapon' in the phytochemical world. One promising therapeutic approach for treating skin wound is to

incorporate natural plant extract like EOs which is probably due to its increased skin vehicle partitioning by the oils. An EO is a concentrated hydrophobic liquid aroma compound containing major active components including hydrocarbons (terpenes and sesquiterpenes) and oxygenated compounds (alcohols, esters, aldehydes, ketones, phenols and ethers) which are known to be associated with many therapeutics activity [5].

According to some researches on several medicinal plants, EOs containing active ingredients mostly from terpenes like eucalyptol, thymol, carvacrol, and caryophyllene have been experimented to accelerate the wound healing process [6–8]. The EO extracts had also shown antioxidant, antimicrobial and anti-inflammation activities through in-vitro studies. Previous study shown that lupeyl acetate, which is a triterpenes was the most abundant component of SrB-EO [9, 10]. Triterpenes have the ability to speed up the wound healing process by accelerating the re-epithelialisation and formation of collagen [11]. For topical uses, EO is able to permeate the membranes including skin owing to its micro size. Nevertheless, EO can be irritant to the skin especially in concentrated condition because it is floating on the water surface. Direct application of EO may cause allergic dermatitis, skin and mucous irritation [12]. Hence, the present study aimed to investigate the further investigation to identify the effective dose of those bioactive compounds which contribute to possible mechanism of action in wound healing.

MATERIAL AND METHODS

Materials

All the chemicals used in this study were analytical grade, unless otherwise specified. Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (MTS reagent) was obtained from Promega (Madison, USA). 10X Phosphate buffer saline (PBS), Dulbecco's Modified Eagle Medium (DMEM) F-12; High glucose (Gibco 12400-016), DMEM with no phenol red; High glucose (Gibco 31053-028), Trypsin EDTA; 0.25%; phenol red (Gibco 25200-056), fetal bovine serum (FBS) (Gibco 10270-098) and Penicillin-streptomycin (Gibco 15140122) were purchased from Thermo Fisher Scientific (MA, USA). Trypan blue solution was purchased from Sigma Aldrich (MO, USA). Gram-positive strains (*Bacillus subtilis* B29 and *Staphylococcus aureus* ATCC 43300), two Gram-negative bacteria (*Escherichia coli* UPMC 25922 and *Pseudomonas aeruginosa* ATCC 15422), one species of fungi, *Candida albicans* ATCC 90028, and Mueller-Hinton agar (MHA), Mueller-Hinton broth and Potato dextrose agar (PDA) were procured from the Institute of Biosciences, UPM. Ethanol, methanol, sodium nitrite, phosphoric acid, and sulphanilamide were purchased from R&M Chemicals (Selangor, Malaysia). RAW264.7 murine macrophages, Hs27 human skin fibroblast and lipopolysaccharides (LPS) of *E. coli* were provided by the Immunology Laboratory, Faculty of Medicine and Health Sciences, UPM.

Figure 1 showed the flow chart of all process that were conducted in this study which include the extraction of SrB-EO, followed by the in vitro cell viability assay on SrB-EO (trypan blue exclusion and MTS assays), and in vitro biological assay on SrB-EO (anti-inflammatory and antimicrobial assays)

Extraction of SrB-EO

SrB dried leaves were obtained from Koperasi Warisan Munsyi Selangor (KOWARIS) Berhad on 19 November 2019. EO from ground SrB dried leaves was prepared using Soxhlet apparatus. A sample of 15.0 g dried SrB leaves were put into a thimble and transferred into a Soxhlet extractor, and the oil was extracted with 200 mL of ethanol at 80ºC and above for 10 cycles [10]. Then, the products were concentrated using a rotary evaporator at 78ºC. A greenish oil crude was obtained. The oil crude (Figure 2) was stored at -20ºC until further use.

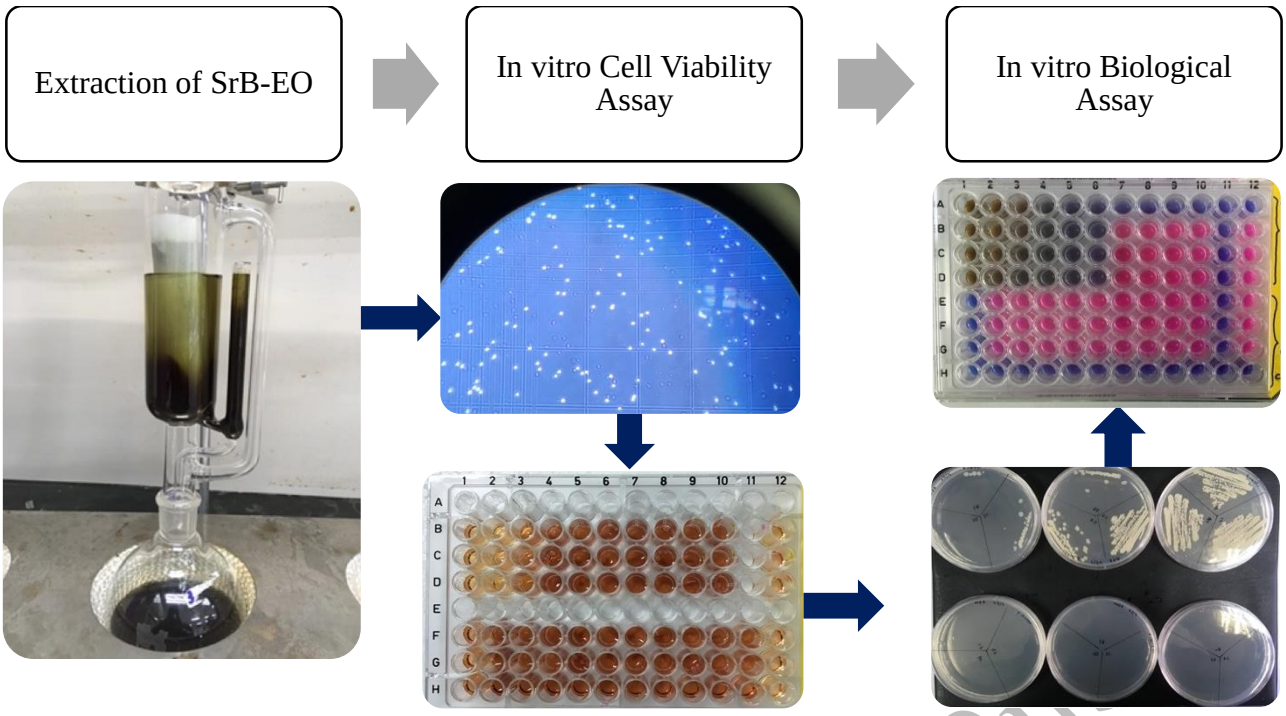

Fig.1 The flow chart of the process conducted in this study

Fig. 2 The greenish oil extracted from SrB dried leaves using Soxhlet method in absolute ethanol

In vitro Cell Viability Assay

Trypan Blue Exclusion Assay

The effect of SrB-EO on cell proliferation activity was evaluated using Hs27 human skin fibroblast cell. The cell's confluency and morphology were observed under light microscope. Cells with 80% confluency were passaged and seeded on 12-well plate at approximately 1.0×10^5 cells per well. The cell cultures were then incubated at 37°C in CO_2 for 24 hours. The cells were then exposed to media alone (DMEM; 10% FBS), solvent (0.1% v/v ethanol) with media, as control, as well as the SrB-EO at concentrations of 50-500 µg/mL. Upon exposure for 24 h, all media from the wells were removed and, cell were washed with PBS (1×, pH 7.4). Cells were then trypsinised and cell count was performed using 0.4% trypan blue using haemocytometer. The principle of trypan blue assay was the live cells with intact cell membranes would exclude this dye, while the dead cells did not. Viable cells will have a clear cytoplasm while the dead cells would have a blue cytoplasm [13].

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

The direct contact method using Hs27 was used to test the in vitro cytotoxicity of the SrB-EO. The confluent cells were reseeded onto the 96-well plate for the cytotoxicity assay at concentration of approximately 5.0 x 10^3 cells per well. Cells were then incubated at 37°C in CO_2 for 24 h and exposed to media alone (DMEM; 2% FBS), solvent (0.1% v/v ethanol) with media, and SrB-EO with media at concentrations of 100-1000 µg/mL for 24 h. Following 24 h of incubation, all media were removed and replaced, and a 20 µL of MTS reagent with media was added into each well was further incubated for 1 hour. Absorbance was recorded at 492 nm, and cell viability was calculated as percentage of viability.

In vitro Biological Studies

Anti-inflammatory Nitrite Assay

The assay employed to assess the potential anti-inflammatory activity of molecules consisted of evaluating their capacity to inhibit nitric oxide (NO) production in activated macrophages [14]. The RAW264.7 were used this assay and the cell's confluency and morphology were confirmed by microscopy. Cells with 80% confluency were seeded on to 24-well plate for the cytotoxicity assay at concentration of approximately 5.0 x 10^4 cells/well with phenol red-free DMEM media containing 10% FBS and 1% antibiotics (penicillin-streptomycin). The cell cultures were then incubated at 37°C for 24 h were induced by 1.0 µg/mL LPS from *E. coli* for 2 hours. Then, the cells were exposed to media alone (phenol red-free DMEM; 2% FBS), solvent (0.1% v/v ethanol) with media, and SrB-EO with media at concentrations 100-500 µg/mL for 24 h. After 24 hours, a 200 µL of aliquot were collected from every well and stored in freezer until further used. The released nitrite ($\mathbf{NO_2^-}$) in the culture medium was measured as an indicator of NO production according to the colorimetric test based on the Griess reaction. Briefly, 50 µL of collected sample was mixed with 50 µL of Griess reagent at room temperature for 30 min. The nitrite concentration was determined by measuring the absorbance at 540 nm using a standard curve of $NaNO_2$ and the absorbance values of all wells were deducted with the absorbance of phenol red-free DMEM medium containing 2% FBS alone, which served as the background reading. The results were expressed as percentage of NO production compared to the control as follows,

$$\%\ \mathbf{Inhibition} = \mathbf{100} \times \frac{[\mathbf{NO_2^-}]\mathbf{exp} - [\mathbf{NO_2^-}]\mathbf{control}}{[\mathbf{NO_2^-}]\mathbf{exp}},$$

With $[\mathbf{NO_2^-}]$control was the concentration of nitrite released without addition of the EO, and $[\mathbf{NO_2^-}$exp the concentration of nitrite released by the cells in presence of the EO.

Antimicrobial Assay

Having shown anti-inflammatory activity, the EOs' antimicrobial activity was evaluated against five different microorganisms namely *B. subtilis* B29, *S. aureus* ATCC 43300, *E. coli* UPMC 25922, *P. aeruginosa* ATCC 15422, and *C. albicans* ATCC using agar Kirby-Bauer disk-diffusion method with no replicate. Test and control microplates were incubated for 24 h at 37 °C for bacteria and 48 h at 25 °C for fungi. Streptomycin (10^8=0.5 McFarland turbidity) and Nystatin (10^8=0.5 McFarland turbidity) were used as positive controls for antibacterial and antifungal assays, respectively.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory concentration (MIC) was determined using the microdilution method in 96-well plates, as described by Ericsson and Sherris (1971) with minor modifications. The SrB-EO were diluted serially at concentration of 0.19–100 mg/mL in absolute ethanol. Absolute ethanol is used as the negative control. Then, 10 µL of each concentration of SrB-EO and absolute ethanol was added to 170 µL of Mueller-Hinton broth in wells of a 96-well microtiter plate, and then 20 µL of standardized suspension of the test organism was added to each well. After incubation for 24 h at 37°C, 30 µL of rezasurin was added into each well and further incubated for 2-4 h for the observation of colour changes. Following incubation, wells with no colour change (blue resazurin colour remained unchanged) were scored as above the MIC value. The lowest concentration of EO at which no visible growth of microorganisms was observed after the incubation period was defined as the MIC. The MIC was confirmed by streaking a swab with the broth from the well that defined as the MIC on Mueller-Hinton agar to observed the growth of microorganism. The minimum bactericidal concentration (MBC) was determined by plating directly the content of wells with higher concentration of sample than the MIC value.

Statistical Analysis

Results were expressed as mean ± standard deviation (n=3). Data analysis was performed using Microsoft Excel 2021 (Washington, USA) and GraphPad Prism version 7 (San Diego, California).

RESULTS

In vitro Cell Viability Assay

Trypan Blue Exclusion Assay

Based on the Figure 3, the results showed the cell viability of Hs27 cells with different concentrations of treatment using 0.4% trypan blue qualitatively within 24 hours. At 100 µg/mL of treatment, the percentage of viable cells reduced to 80% for Hs27 compared with trypan blue free control group (cell only). At 250 µg/mL of treatment, the percentage of viable cells reduced about 27% for and Hs27 compared with trypan blue free control group. On the other hand, 500 µg/mL, the cells show elevation of the percentages of viable cells compared with trypan blue free control group.

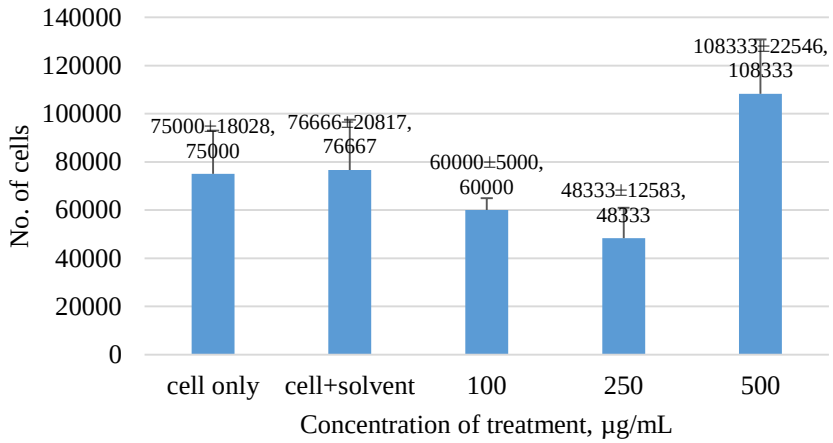

Fig. 3 Dose-dependent Trypan Blue Assay of SrB-EO on Hs27 after 24 hours treatment and incubated in DMEM containing 2% FBS at 37°C CO^2. Data label: mean±standard deviation (SD).

MTS Assay

The quantitative cell viability assay of the EO studied using Hs27 cells was evaluated with the Cell Titer 96 (MTS reagent) after 24 hours treatment of the cell with the different concentrations of treatment given in Figure 4. Hs27 cell is sensitive to the toxic effect of SrB-EO, with IC_{50} value of 735.5 µg/mL. It showed that the cell viability of cells decreased when the cells were exposed to higher concentration of EO. The highest concentration tested (1000 µg/mL) decreased cell viability by 82.02% on Hs27 cells. The cytotoxicity assay SrB-EO upon Hs27 cells started to show decreasing cell viability for concentration equal or greater than 800 µg/mL.

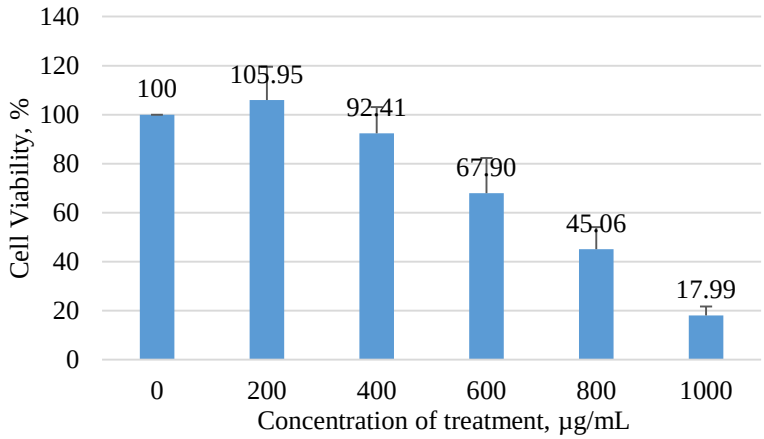

Fig. 4 Dose-dependent MTS Assay of SrB-EO on Hs27 using after 24 hours treatment and incubated in DMEM containing 2% FBS at 37°C CO^2

In vitro Biological Studies

Anti-inflammatory Nitrite Assay

The potential anti-inflammatory activity of SrB-EO were investigated using RAW 264.7 murine macrophages cells. Cells were induced by 1 µg/mL LPS for 2 hours and the effects of EO during co-incubation period of 24 h was determined by using NO production as results parameter. The assays were validated by using non-induced cells as negative control and LPS-induced cells as positive control. As shown in Figure 5, the SrB-EO significantly reduced the stimulated NO production in a dose-dependent manner of 33.12% at the highest tested concentration of 500 µg/mL, meanwhile, 17.02% and 2.7% inhibition of NO production in macrophages stimulated with LPS at 250 µg/mL and 100 µg/mL, respectively.

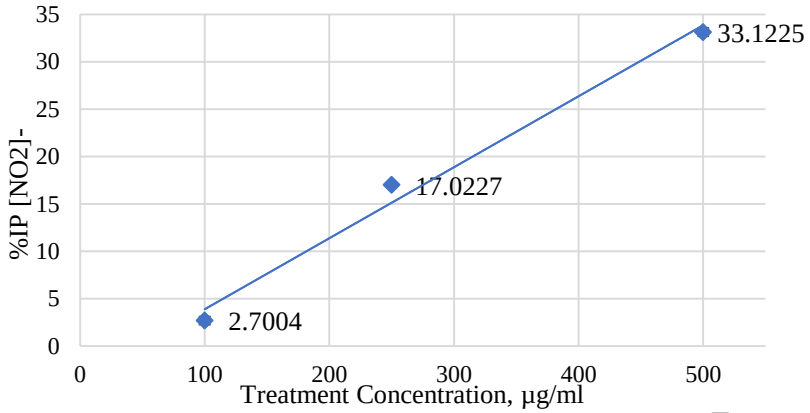

Fig. 5 The relationship between the inhibition percentage (IP) of NO_2^- and the concentration of treatment (SrB-EO) after RAW264.7 cells induced by 1.0 µg/mL of LPS for 2 h at 37°C CO^2

Antimicrobial Assay

Among three groups of microbes which Gram-positive bacteria, Gram-negative bacteria and fungi, SrB-EO showed significant antibacterial activity against Gram-positive bacteria (Figure 6). The largest zone of inhibition was observed for SrB-EO against *S. aureus* (12 mm) and inhibition zone of EO against *B. subtilis* was 7 mm (Table 1). According to previous studies, diameters of inhibition zone (DIZ) were valued as follows: Not sensitive (diameter ≤ 8.0 mm), moderately sensitive (8.0 < diameter < 14.0 mm), sensitive (14.0 < diameter < 20.0 mm), and extremely sensitive (diameter ≥ 20.0 mm) [16]. Therefore, SrB-EO showed negative antimicrobial activity against tested Gram-negative bacteria and fungi.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

As shown in Table 2 and Figure 7, the MICs revealed that SrB-EO exhibited antibacterial activities against both Gram-positive bacteria; *S. aureus* (MIC:3.13 mg/mL) and *B. subtilis* (MIC:6.25 mg/mL). The MBCs of SrB-EO were 10.42 mg/mL and 12.5 mg/mL against *S. aureus* and *B. subtilis*, respectively.

Table 1 The range of zone diameters for SrB-EO against Gram-positive bacteria (*B. subtilis* and *S. aureus*), Gram-negative bacteria (*E.coli* and *P. aeruginosa*), and Fungi (*C. albicans*)

Sample(s)	*B. subtilis* B29	*S. aureus* ATCC 43300	*E. coli* UPMC 25922	*P. aeruginosa* ATCC 15422	*C. albicans* ATCC 90028
SrB-EO (mm)	7	12	n.a	n.a	n.a
Streptomycin (mm)	28	27	28	28	-
Nystatin (mm)	-	-	-	-	32

Table 2 The MIC and MBC of SrB-EO against microbes

Sample(s)	*B. subtilis* B29	*S. aureus* ATCC 43300
MIC (mg/mL)	6.25	3.13
MBC (mg/mL)	12.5	10.42

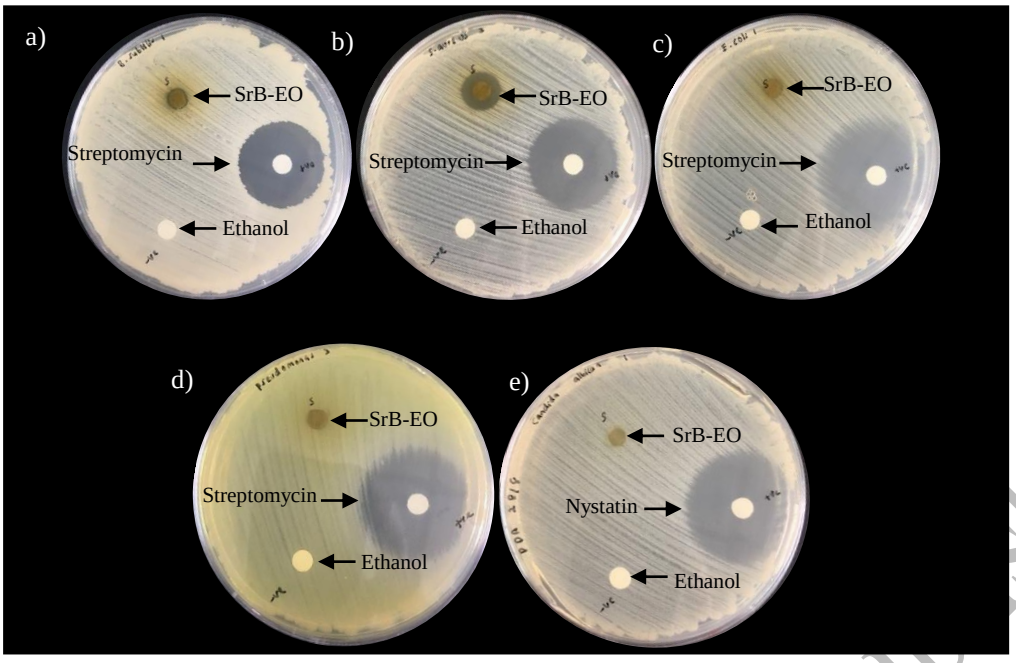

Fig. 6 Disk diffusion method of 100 mg/mL SrB-EO extract using; a) *B. subtilis*, b) *S. aureus*, c) *E. coli*, d) *P. aeruginosa*, on MHA and e) *C. albicans* on PDA as test microorganisms

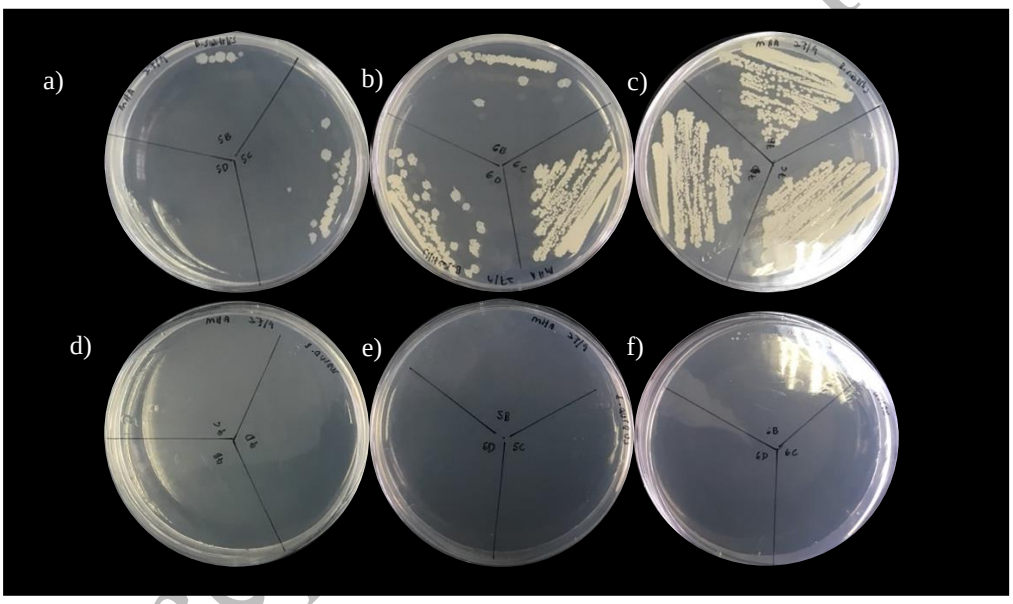

Fig. 7 Streak method of SrB-EO extract of; a) 6.25 mg/mL, b) 3.13 mg/mL, and c) 1.56 mg/mL using *B. subtilis* as test microorganism and d) 12.5 mg/mL, e) 6.25 mg/mL and f) 3.13 mg/mL using *S. aureus* as test microorganism on MHA

DISCUSSION

In vitro Cell Viability Assay

Cell viability assay is one of the most significant characterizations for measuring therapeutic properties in cell. The importance of cell viability and cell counting for cell and gene therapy has been acknowledged by the U.S. Congress in the 21st Century Cure Act [17]. There are two assays used in this study; trypan blue dye exclusion assay and MTS colorimetric test. The trypan blue assay is a quantitative assay which indicates only cell is alive while MTS assay is quantitative assay which the growth and death rate can be measured [18–20].

Overall, all concentration of treatments except 1mg/mL of SrB-EO showed negligible level of cytotoxicity. However, trypan blue dye exclusion cannot be applied to differentiate between healthy cells and the cells that alive but malfunction. Furthermore, generally trypan blue staining cell count done using haemocytometer, thus errors in counting, dilution of cells, and improper handling the chamber might happen during the processes. This

procedure is not sufficiently sensitive for in vitro cytotoxicity assay [13]. Thus, another cell viability test needed for accurate result.

According to ISO 10993-5, percentages of cell viability above 80% are considered as non-cytotoxicity; within 60% to 80% are weak; 40% to 60% are moderate and below 40% are strong cytotoxicity, respectively [21]. Thus, a concentration of 500 μg/mL was chosen as the concentration to be used in anti-inflammatory assay using RAW264.7 using Hs27.

In vitro Biological Studies

Medicinal plants have proven their value as sources of molecules with therapeutic potential. Nevertheless, EO as the plant secondary metabolites possesses various biological activities like antioxidant, anti-inflammatory, and antimicrobial which are powerful candidates for developing new phytopharmaceuticals [22].

Inflammation is a biological protective response which is induced by the microbial invasion or tissue injury present within the body and responsible in removing damaged or dead host cells. Inflammation stimulates the metabolism of arachidonic acid and the activity of various enzymes including nitric oxide synthases (iNOS), oxygenases, and peroxidases [23]. Inflammation induced by nitric oxide synthase has become an important pharmacological target since overproduction of NO after induction of this enzyme seems to be linked with various pathological circumstances. NO is a free radical with principal complex regulatory activity on the functions, growth and death of many cell types involved in immune and inflammatory response [24]. The NO released from cells are observed and quantified using a colorimetric reaction using Griess reagent, photometrically [25].

Microorganisms such as bacteria, viruses, fungi and protozoa are the aetiological factors of many infectious diseases, and the compounds with antimicrobial activity are the best weapon for treating these diseases. The method commonly used in investigating antibacterial activity is disc diffusion method, which the activity are observed in the form of zone of inhibition. Then, serial dilution test using microdilution with a known concentration of extract can be used to determine minimum inhibitory concentrations (MIC) [26]. The MIC is defined as the lowest concentration of EO required to inhibit the visible growth of the microorganism. The microdilution of EO also can be used to quantify the surviving organisms using resazurin as indicator which allows for the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) to be determined. The MBC and MFC refer to the minimum concentration of EO required to kill 99.9% of the cells originally inoculated into the assay for bacteria and fungi, respectively [27].

According to our previous study, the presence of terpenes and phenols in the EO seems to responsible for the anti-inflammatory activity [10, 28]. The SrB-EO was reported to own about 36% of terpenes, which lupeol acetate was the highest terpenes group and about 8% of phenols. The presence of terpene compounds found in SrB-EO like chavibetol, eugenol, and lupeol [10] were also observed by other study to possess a potential anti-inflammatory properties [29–31]. In spite of that, the anti-inflammatory activity and the radical-scavenging activity were found to be positively correlated, thus demonstrating the highest antioxidant activity was correlated with the highest total phenolic contents and/or the highest anti-inflammatory activity of the tested extract [14]. Furthermore, the anti-inflammatory activity of EO not only ascribed due to the their radical-scavenging activities, but also to their interactions with signalling cascades involving cytokines and regulatory transcription factors, and on the expression of pro-inflammatory genes [32].

Some EO owns antibacterial activity, but no antifungal activity, while some have strong antifungal activity but no antibacterial activity [33]. As reported by Valerio *et al*. (2021), the EO extracted from *Salvia fruticose* subsp. thomasii sourced from two different location possessed antibacterial activity but did not show antifungal activity. At the same study, EO from *Satureja montana* subsp. montana (Sm1 and Sm2) of two location show opposite behaviour, which Sm2 show antifungal activity but Sm1 did not show any antifungal activity [34]. Compounds that responsible for this antibacterial properties in SrB-EO were chavibetol and eugenol [35] as reported by Mohamad Zen *et al*. (2019) too.

In general, Gram-negative bacteria are more resistant than Gram-positive bacteria because the cell wall of Gram-negative bacteria is more complex. The structure of cell wall of Gram-positive bacteria allows hydrophobic compounds to penetrate the cell wall and cytoplasm of the cells. The antimicrobial properties which possessed by phenolic compound in EO are hydrophobic [36]. Both tested Gram-positive bacteria are cutaneous microbiota and common found in isolated chronic wound [37, 38], thus demonstrating the potent topical use of SrB-EO.

Based on recent studies on the antibacterial assessment of the EOs from oregano, thyme, cinnamon bark, and lemon grass, they also showed a remarkable activity against tested Gram-positive pathogen. EO can be used topically for antimicrobial purposes, but it must be in diluted form for safe application due to its toxicity [39].

CONCLUSION

In conclusion, the cell viability and biological activities of SrB-EO were investigated. The concentration of SrB-EO with very low toxicity toward human skin cells was identified (500 μg/mL) and possessed promising anti-inflammatory activity. Also, the SrB-EO showed satisfactory antibacterial activity against tested Gram-positive. Thus, SrB-EO can be used as alternative therapeutic agents for topical application in pharmacological and medicinal field.

Statements and Declarations

The authors declare that they have no competing interests

Author Contributions

Conceptualization, Nur Izzati Mohamad Zen and Uswatun Hasanah Zaidan; Data curation, Suhaili Shamsi; Formal analysis, Nur Izzati Mohamad Zen; Funding acquisition, Uswatun Hasanah Zaidan and Nazrim Marikkar; Investigation, Nur Izzati Mohamad Zen; Methodology, Nur Izzati Mohamad Zen and Masriana Hassan; Project administration, Uswatun Hasanah Zaidan; Resources, Siti Salwa A. Gani; Software, Nur Izzati Mohamad Zen; Supervision, Uswatun Hasanah Zaidan; Validation, Uswatun Hasanah Zaidan, Suhaili Shamsi and Masriana Hassan; Writing – original draft, Nur Izzati Mohamad Zen; Writing – review & editing, Uswatun Hasanah Zaidan.

ACKNOWLEDGMENTS

The authors wish to thank Laboratory of Food and Microbiome Technology Laboratory (FAMTech), Faculty of Biotechnology and Biomolecular Sciences and Immunology Laboratory, Faculty of Medicine and Health Sciences for providing the useful knowledge and required facilities during conducting the research. This work has been supported by research grant from Universiti Putra Malaysia (GP/2018/9631600).

REFERENCES

1. Ambrose D.C.P., Manickavasagan A, Naik R. Leafy Medicinal Herbs: Botany, Chemistry, Postharvest Technology and Uses. CABI; 2016:xiii + 282 pp. doi:10.1079/9781780645599.0000
2. Orellana-Paucar A.M. Steviol Glycosides from *Stevia rebaudiana*: An Updated Overview of Their Sweetening Activity, Pharmacological Properties, and Safety Aspects. Molecules. 2023;28(3):1258. doi:10.3390/molecules28031258
3. Baser K.H.C., Buchbauer G. Handbook of Essential Oils: Science, Technology, and Applications. CRC Press; 2020.
4. Padalia R.C., Verma D.K., Arora C., Mahish P.K. Essential Oils: Sources, Production and Applications. Walter de Gruyter GmbH & Co KG; 2023.
5. Aggarwal S., Agarwal S., Jalhan S. Essential oils as novel human skin penetration enhancer for transdermal drug delivery: A review. Int. J. Pharm. Biol. Sci. 2013;4(1):857-868.
6. Costa M.F., Durço A.O., Rabelo T.K., Barreto R. de S.S., Guimarães A.G. Effects of Carvacrol, Thymol and essential oils containing such monoterpenes on wound healing: a systematic review. J. Pharm. Pharmacol. 2019;71(2):141-155. doi:10.1111/jphp.13054
7. Arooj B., Asghar S., Saleem M., Khalid S.H., Asif M., Chohan T., Khan I.U., Zubair H.M., Yaseen H.S. Anti-inflammatory mechanisms of eucalyptol rich *Eucalyptus* globulus essential oil alone and in combination with flurbiprofen. Inflammopharmacology. 2023;31(4):1849-1862. doi:10.1007/s10787-023-01237-6
8. Koyama S., Purk A., Kaur M., Soini H.A., Novotny M.V., Davis K., Kao C.C., Matsunami H., Mescher A. Beta-caryophyllene enhances wound healing through multiple routes. PloS One. 2019;14(12):e0216104. doi:10.1371/journal.pone.0216104
9. Mohamad Zen N.I., Zaidan U.H., Abd Gani S.S., Shamsi S., Halmi M.I.E. Essential Oil Constituents Assessment and Antioxidant Profiles of *Stevia rebaudiana* Bertoni Leaves from Malaysia. Malays. Appl. Biol. 2019;48:55-62.
10. Zaidan U.H., Hamid S.N.M., Mat Yusof M.F., Ahmad S., Shamsi S. Chemical Evaluation And Antioxidant Properties of Extracts and Essential Oil from *Stevia rebaudiana* Leaves. Malays Appl Biol. 2018;47(2):15-23.
11. Ghiulai R., Roşca O.J., Antal D.S., Mioc M., Mioc A., Racoviceanu R., Macaşoi I., Olariu T., Dehelean C., Creţu O.M., Voicu M., Şoica C. Tetracyclic and Pentacyclic Triterpenes with High Therapeutic Efficiency in Wound Healing Approaches. Molecules. 2020;25(23):5557. doi:10.3390/molecules25235557

12. Bensouilah J., Buck P. Aromadermatology: Aromatherapy in the Treatment and Care of Common Skin Conditions. Routledge; 2021.
13. Aslantürk Ö.S. In Vitro Cytotoxicity and Cell Viability Assays: Principles, Advantages, and Disadvantages. In: Genotoxicity - A Predictable Risk to Our Actual World. InTech; 2018. doi:10.5772/intechopen.71923
14. Muanda F.N., Soulimani R., Diop B., Dicko A. Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves. LWT - Food Sci. Technol. 2011;44(9):1865-1872. doi:10.1016/j.lwt.2010.12.002
15. Ericsson H.M., Sherris J.C. Antibiotic sensitivity testing. Report of an international collaborative study. Acta Pathol. Microbiol. Scand. [B] Microbiol. Immunol. 1971;217:Suppl 217:1+-Suppl 217:1+.
16. Li Z.H., Cai M., Liu Y.S., Sun P.L., Luo S.L. Antibacterial Activity and Mechanisms of Essential Oil from *Citrus medica* L. Var. *Sarcodactylis.* Molecules. 2019;24(8). doi:10.3390/molecules24081577
17. Chan L.L.Y., Rice W.L., Qiu J. Observation and quantification of the morphological effect of trypan blue rupturing dead or dying cells. PLoS One. 2020;15(1). doi:10.1371/journal.pone.0227950
18. Babakhanova G., Zimmerman S.M., Pierce L.T., Sarkar S., Schaub N.J., Simon C.G.Jr. Quantitative, traceable determination of cell viability using absorbance microscopy. PLoS One. 2022;17(1):e0262119. doi:10.1371/journal.pone.0262119
19. Kamiloglu S., Sari G., Ozdal T., Capanoglu E. Guidelines for cell viability assays. Food Front. 2020;1(3):332-349. doi:10.1002/fft2.44
20. Demetzos C., Pippa N. Thermodynamics and Biophysics of Biomedical Nanosystems: Applications and Practical Considerations. Springer; 2019.
21. International Standard I. ISO 10993-5:2009 - Biological evaluation of medical devices- Part 5: Tests for in vitro cytotoxicity. Published online 2009.
22. Toiu A., Mocan A., Vlase L., Pârvu A.E., Vodnar D.C., Gheldiu A.M., Moldovan C., Oniga I. Phytochemical Composition, Antioxidant, Antimicrobial and *in vivo* Anti-inflammatory Activity of Traditionally Used Romanian *Ajuga laxmannii* (Murray) Benth. ("Nobleman's Beard" - Barba Împăratului). Front. Pharmacol. 2018;9(MAR):7-7. doi:10.3389/fphar.2018.00007
23. Malik S., ed. Essential Oil Research: Trends in Biosynthesis, Analytics, Industrial Applications and Biotechnological Production. Springer, 2019.
24. Benevides Bahiense J., Marques F.M., Figueira M.M., Vargas T.S., Kondratyuk T.P., Endringer D.C., Scherer R., Fronza M. Potential anti-inflammatory, antioxidant and antimicrobial activities of *Sambucus australis*. Pharm. Biol. 2017;55(1):991-997. doi:10.1080/13880209.2017.1285324
25. V. Pinto R., Antunes F., Pires J., Silva-Herdade A., Pinto M.L. A Comparison of Different Approaches to Quantify Nitric Oxide Release from NO-Releasing Materials in Relevant Biological Media. Molecules. 2020;25(11):2580. doi:10.3390/molecules25112580
26. Domb A.J., Kunduru K.R., Farah S. Antimicrobial Materials for Biomedical Applications. Royal Society of Chemistry; 2019.
27. Sateriale D., Forgione G., De Cristofaro G.A., Facchiano S., Boscaino F., Pagliuca C., Colicchio R., Salvatore P., Paolucci M., Pagliarulo C. Towards Green Strategies of Food Security: Antibacterial Synergy of Essential Oils from *Thymus vulgaris* and *Syzygium aromaticum* to Inhibit *Escherichia coli* and *Staphylococcus aureus* Pathogenic Food Isolates. Microorganisms. 2022;10(12):2446. doi:10.3390/microorganisms10122446
28. Zaidan U.H., Mohamad Zen N.I., Amran N.A., Shamsi S., Abd Gani S.S. Biochemical evaluation of phenolic compounds and steviol glycoside from *Stevia rebaudiana* extracts associated with in vitro antidiabetic potential. Biocatal. Agric. Biotechnol. 2019;18:101049-101049. doi:10.1016/J.BCAB.2019.101049
29. Liu K., Zhang X., Xie L., Deng M., Chen H., Song J., Long J., Li X., Luo J. Lupeol and its derivatives as anticancer and anti-inflammatory agents: Molecular mechanisms and therapeutic efficacy. Pharmacol. Res. 2021;164:105373. doi:10.1016/j.phrs.2020.105373
30. Pawar S., Bhane P., Jha U. Review on Betel Pepper Leaf for Anti Inflammatory Activity. World J. Pharm. Res. 2021;11(2):1196-1208.
31. Barboza J.N., da Silva Maia Bezerra Filho C., Silva R.O., Medeiros J.V.R., de Sousa D.P. An Overview on the Anti-inflammatory Potential and Antioxidant Profile of Eugenol. Oxid. Med. Cell. Longev. 2018;2018:3957262. doi:10.1155/2018/3957262
32. Miguel M.G. Antioxidant and anti-inflammatory activities of essential oils: A short review. Molecules. 2010;15(12):9252-9287. doi:10.3390/molecules15129252
33. Lis-Balchin M. Aromatherapy Science: A Guide for Healthcare Professionals. 1st ed. Pharmaceutical Press; 2006:446. doi:10.1111/j.2042-7166.2006.tb04705.x

34. Valerio F., Mezzapesa G.N., Ghannouchi A., Mondelli D., Logrieco A.F., Perrino E.V. Characterization and Antimicrobial Properties of Essential Oils from Four Wild Taxa of *Lamiaceae* Family Growing in Apulia. Agronomy. 2021;11(7):1431. doi:10.3390/agronomy11071431
35. Tran V.T., Nguyen T.B., Nguyen H.C., Do N.H.N., Le P.K. Recent applications of natural bioactive compounds from *Piper betle* (L.) leaves in food preservation. Food Control. 2023;154:110026. doi:10.1016/j.foodcont.2023.110026
36. Andrade-Ochoa S., Chacón-Vargas K.F., Sánchez-Torres L.E., Rivera-Chavira B.E., Nogueda-Torres B., Nevárez-Moorillón G.V. Differential Antimicrobial Effect of Essential Oils and Their Main Components: Insights Based on the Cell Membrane and External Structure. Membranes. 2021;11(6):405. doi:10.3390/membranes11060405
37. Byrd A.L., Belkaid Y., Segre J.A. The human skin microbiome. Nat. Rev. Microbiol. 2018;16(3):143-155. doi:10.1038/nrmicro.2017.157
38. Hernandez-Valdes J.A., Zhou L., de Vries M.P., Kuipers O.P. Impact of spatial proximity on territoriality among human skin bacteria. NPJ Biofilms Microbiomes. 2020;6(1):1-13. doi:10.1038/s41522-020-00140-0
39. Patterson J.E., McElmeel L., Wiederhold N.P. In Vitro Activity of Essential Oils Against Gram-Positive and Gram-Negative Clinical Isolates, Including Carbapenem-Resistant *Enterobacteriaceae*. Open Forum Infect Dis. 2019;6(12):ofz502. doi:10.1093/ofid/ofz502