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Biological properties and active components of *Ocimum*

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Abstract

Ocimum sanctum Linn. and *Ocimum gratissimum Linn.* have been used for traditional and ayurvedic purposes for centuries. These aromatic plants have also been recognized as having potential medicinal properties and studied for their anti-inflammatory and anti-bacterial properties. The aims of this study were to prepare ethanol extracts from the leaves of *O. sanctum* and *O. gratissimum* and characterize the bioactive components and to determine their cytotoxic effects of on L929 mouse fibroblast cell line. Fresh leaves of the plants were macerated and the extracts were obtained using 95% ethanol. Fourier transmission infra-red spectrum of both herbal extracts were analyzed to identify the functional groups. The intensity peaks were indicative of aliphatic primary amines, aromatic compounds, alkanes, and alkyl halides. Murine fibroblast cells were then treated with 9 different extract concentrations ranging from 60 mg/mL to 0.23 mg/mL, following two-fold dilution. Cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for both plant extracts using ISO 10993-5. The results showed that 95% ethanol extracts of *Ocimum sanctum* and *Ocimum gratissimum* at the concentrations of ≤0.46 mg/mL had no toxic effects on L929 cell lines.

Keywords: Active component, Cytotoxicity test, *Ocimum*

1. Introduction

Ocimum (basil) is the most important genus of the family of Lamiaceae, which is a large family of aromatic herbs and shrubs [1]. *Ocimum sanctum* Linn. is known colloquially as “the queen of herbs”, “holy basil”, “sacred basil”, or “ka-praw” in Thai. It is a branched sub-herb that grows to a height of 30-60 cm with green to purple leaves. The leaves are ovate and generally grow up to 5 cm in length (Figure 1 (A)) [2]. It is found throughout the world and is known to have multiple medicinal properties. All parts of the plant are considered beneficial in traditional medicine but fresh or dried leaves are used most commonly either alone or in combination with other herbs. It is used as a remedy for the common cold, headaches, cough, malaria, dengue, stomach disorders, eye diseases, mouth infections, insect bites, inflammation, heart diseases, and various forms of poisoning [3].

Ocimum gratissimum Linn. also belongs to the same family “Lamiaceae”. It is commonly known as “alfavaca”, “tree basil”, or “vana tulsi” and in Thailand it is called “yee-rah”. It is a shrub that grows to a height of 1.9 m with leaves that are ovate to ovate-lanceolate, measuring up to 10 x 5 cm (Figure 1 (B)). It is natural to African Madagascar, Southern Asia, and naturalized in countries such as Mexico, West Indies, and Brazil [3]. Traditionally, it is used as a medical herb for the treatment of many diseases such as fever, constipation, eyes diseases, skin infection, bronchitis, and headaches, and even as an anti-parasitic and anti-diarrheal agent [4].

Several sources in the literature have recommended *O. sanctum* and *O. gratissimum* for their anti-microbial and anti-inflammatory properties, which can promote wound healing after tissue injury [5-8]. Safety is the foremost consideration when using herbal medications. Moreover, there is a lack of pharmacological

information regarding cytotoxicity of these herbs. Therefore, the aims of this study were (1) to prepare ethanol extracts of *O. sanctum* and *O. gratissimum* in order to characterize their bioactive components and (2) to determine cytotoxic effects of these extracts on murine L929 cell line using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

2. Materials and methods

2.1 Plant collection

Leaves of organically grown *O. sanctum* and *O. gratissimum* were obtained from a courtyard in Chonburi province, Thailand. All leaves of the plants were collected for use, except for the first three leaves of the plant, also known as the "seed leaves" or cotyledons.

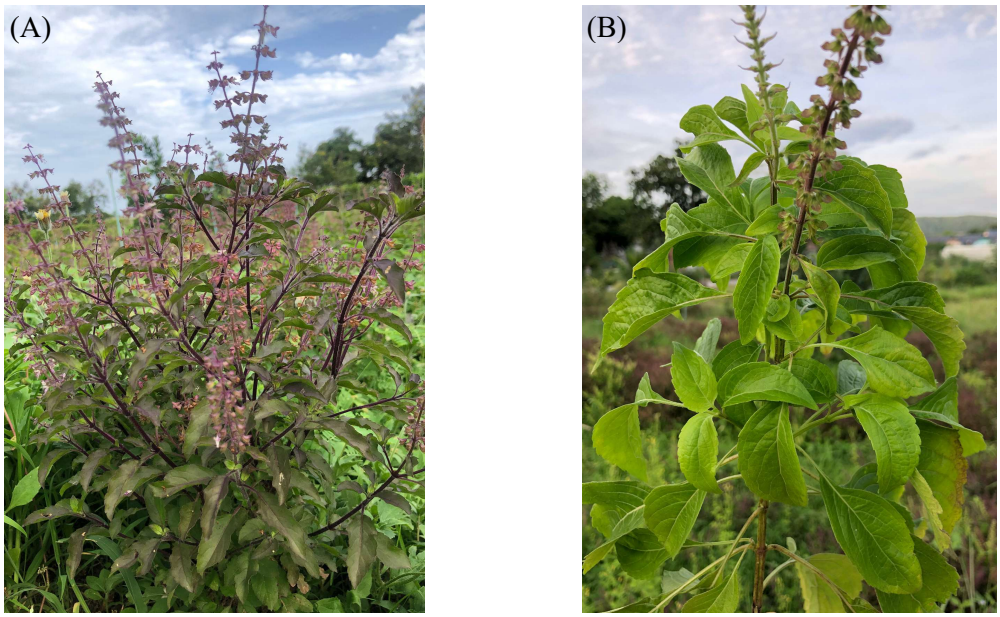


Figure 1 (A) *O. sanctum*, and (B) *O. gratissimum*.

2.2 Sample preparation and crude extraction

Leaves of the herbs were sun-dried in a plastic dome at 54 °C for 2 days using an infra-red light. Then, dried leaves of *O. sanctum* and *O. gratissimum,* weighing 1000 g and 1500 g respectively, were ground and macerated with 3000 mL of 95% ethanol for 7 days at room temperature. The liquid extract was evaporated using a rotary evaporator (Buchi, Switzerland) and powder extract was created using a spray dryer (Buchi mini spray dryer B-290). Then, they were weighed to calculate the final yield. The extracts were sealed in glass bottles and stored in dark conditions before usage.

2.3 Fourier transmission infrared (FTIR) spectroscopy

The organic components present in the extracts were analyzed using an FTIR spectrophotometer (INVENIO-S; Bruker Switzerland AG, Thailand). The samples were placed into an FTIR spectroscope with a scan range of wavenumber 4000 - 400 cm^{-1} with a resolution of 4.0/cm. The spectrum resulting transmission versus wavenumber was observed and compared with the available database.

2.4 Cell line

Murine fibroblast cell line (Lot number: 58494139) was obtained from the cell culture bank of the Research Unit, Faculty of Dentistry, Mahidol University, Thailand. Cells were thawed in a water bath at 37 °C. Then, cells were seeded into cell culture flask 75 cm^2 maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic (penicillin-streptomycin) and incubated in

5% CO_2 atmosphere at 37 °C overnight. The cell culture was examined every day followed by medium change on alternating days. After 70-80% monolayer cell growth occurred, the cells were further sub-cultured.

2.5 Cytotoxicity test

L929 $1x10^4$ cells/well (volume 100 µL) were seeded into 96-well plates and incubated at 5% CO_2 at 37 °C for 24 h until a cell monolayer formed. After incubation, plant extracts were diluted with 10% DMEM at varying concentrations. Culture media was removed from the well-plates and the extract dilutions were added to a final volume of 100 µL/well. The plant extracts were prepared at 6% wt/vol and diluted with 2-fold dilution to 9 concentrations (60, 30, 15, 7.5, 3.75, 1.87, 0.93, 0.46, and 0.23 mg/mL). Both ethanol extracts of *O. sanctum* and *O. gratissimum* samples were prepared separately. A blank control group was prepared using 10% DMEM with the same volume of 100 µL/well and maintained at 5% CO_2 incubator at 37 °C. After 24 h, the culture media was discarded and each well was washed by PBS twice. Fifty µL of MTT solution (1 mg/mL of MTT, 13.4 mg/mL of DMEM, 10% v/v of sodium bicarbonate, and distilled water) was added into each well, including the control group, and incubated in a 5% CO_2 incubator at 37 °C for 2 h. After incubation, MTT was removed and each well was washed by PBS. Then, 100 µL of isopropanol was added into each well to dissolve the MTT formazan. Before determination of cytotoxicity, plates were swayed for 30 min. The spectrophotometric absorbance at 570 nm (reference 650 nm) was studied using a 96-well plate microplate reader (Ceres UV 900 HDi, Biotek Instrument, USA) to determine the cytotoxic effect on the L929 cell line. The assessment of the cytotoxicity of *O. sanctum* and *O. gratissimum* was based on ISO 10933-5 with modification following Archana et al. [9] Cell viability of <70% was considered as threshold value for cytotoxicity. Ten samples were used for each concentration including the control group, and experiments were repeated 3 times for both herbs.

2.6 Cytotoxicity evaluation

Cell survival percentage was calculated based on a formula [10]. The absorbance is directly proportional to the number of living cells. All samples were compared with control to calculate the percentage of vital cells, using the following equation:

$$\% \text{ cell viability} = \frac{OD_{570\ (sample)x100}}{OD_{570\ (control)}} \quad (1)$$

2.7 Statistical analysis

Microsoft Excel 2010 was used for descriptive statistical analysis. The experimental data was expressed as mean ± SD. The significance of the different concentrations of *O. sanctum* and *O. gratissimum* ethanol extract treated groups and control group was assessed by the biological evaluation of medical device – part 5: tests for *in vitro* cytotoxicity, International Standard Organization ISO10993-5. Normal distribution was analyzed with the Kolmogorov–Smirnov test and comparison of each treatment and control group was tested by independent t-test. The level of significance was set at 95% CI (p-value < 0.05)

3. Results

The extracted powder after solvent evaporation appeared as a granular sticky dark brown colored powder for *O. sanctum,* whereas *O. gratissimum* extract appeared as a dry fine olive-colored powder. The final yield of the extract was 5% (w/w) and 4.5% (w/w) for *O. sanctum* and *O. gratissimum,* respectively. The FTIR spectra of leaf extracts from *O. sanctum* (Figure 2 (A)) and *O. gratissimum* (Figure 2 (B)) with peak values indicative of the various functional groups have been summarized in Table 1.

The results of the cytotoxicity test showed that for *O. sanctum* and *O. gratissimum* extract concentrations ≥0.93 mg/mL have cytotoxic effects on the mouse fibroblast cell line when compared to the control group. Extract treated groups had percentage of cell viability of <70% as shown in Figure 3. Concentrations between 0.23 - 0.46 mg/mL had no toxic effects on the mouse fibroblast cell line.

Independent t-test was used to compare between each *O. sanctum* and *O. gratissimum* extract treated and control groups. The result showed significant differences between treatment groups (from 60 mg/mL to 0.93 mg/mL) and the control group. The other groups of both herbs, at the concentrations of 0.23 and 0.46 mg/mL showed no significant differences with the control group (p<0.05) (Figure 3).

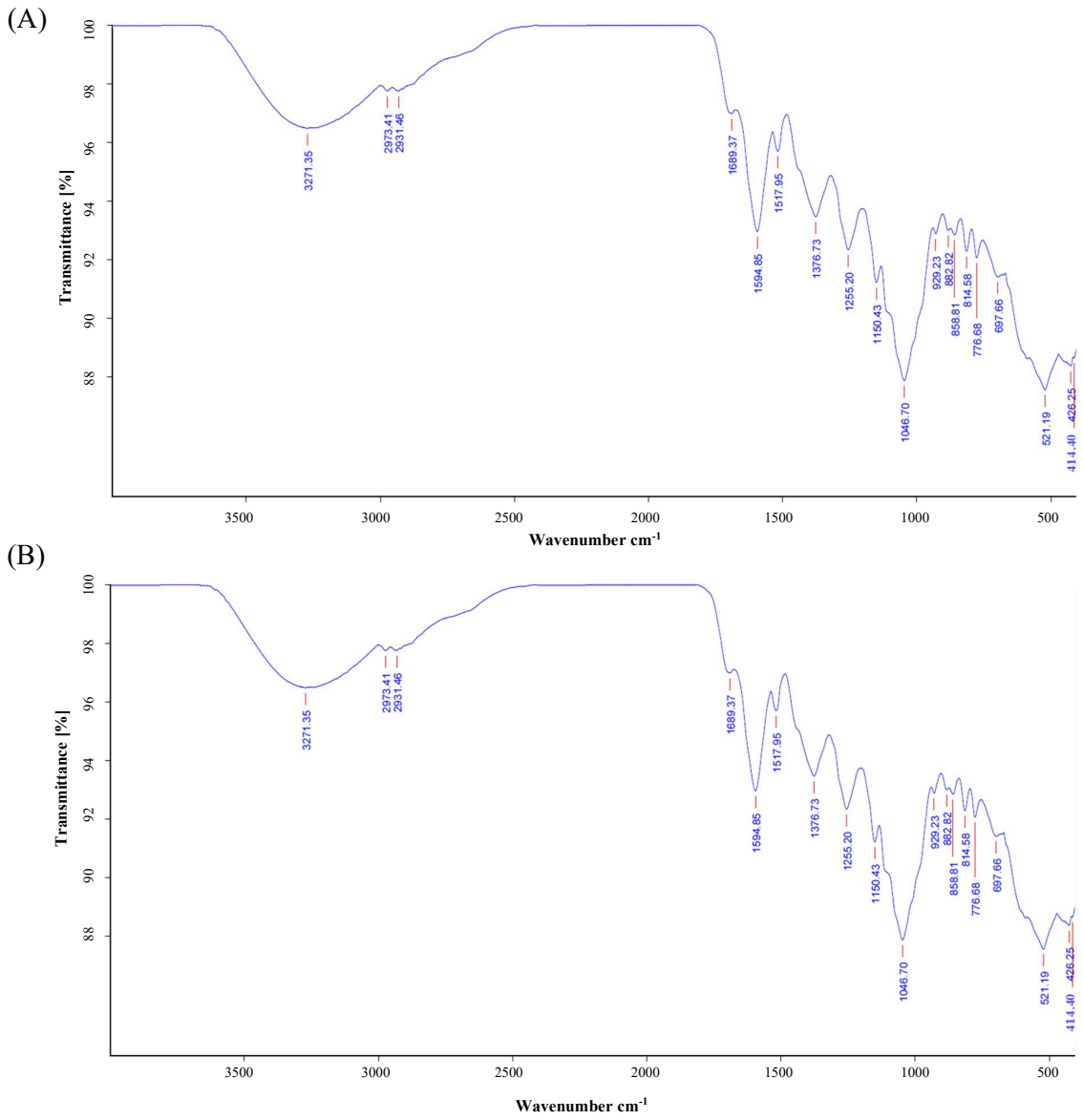
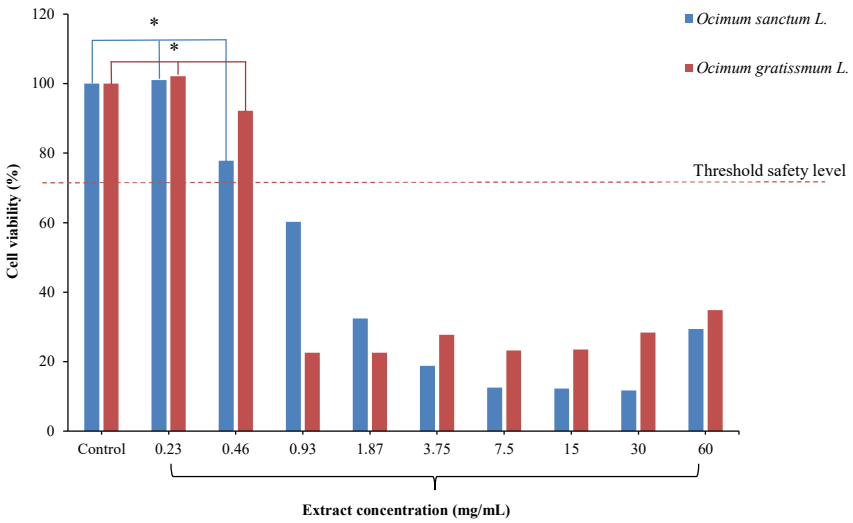


Figure 2 FTIR spectrum of (A) *O. sanctum*, and (B) *O. gratissimum* leaf extract.

Table 1 FTIR spectrum of *O. sanctum and O. gratissimum* extracts with corresponding functional groups.

Wavenumber (cm^{-1})		Group	Functional group
O. sanctum	*O. gratissimum*		
3271.90	3271.35	N-H stretch	Aliphatic primary amine
2933.10	2973.41/2931.46	C-H stretch	Alkanes
1597.63	1594.85	C=C-C	Aromatic ring stretch
1256.13	1255.20	C-F stretch	Alkyl halide
1042.29	1046.70	C-N stretch	Ether
515.90	521.19	C-Br stretch	Alkyl halide



*Not significantly different when compared to the control.

Figure 3 Intra-group comparison of two herbs at different concentrations against the control with threshold safety value set at 70% cell viability.

4. Discussion

Obtaining herb extracts with relatively high yield is a challenging task. Furthermore, different extraction methods will give different total substance yield. In our study, a maceration ethanol extraction technique was followed. This involves the separation of potentially medicinally active compounds from plants by soaking in a solvent. Ethanol and acetone have been suggested as many of the herbal essential oils are soluble in them and preserved during the extraction process [11]. In our experiment, ethanol was used as it is a highly efficient solvent and can easily evaporate from extracts using a spray drying technique. The final yield in our study was in the range of 4.5-5% (w/w) for both herbs.

FTIR spectroscopy is recognized as one of the most popular analytical techniques to identify the type of functional groups of organic compounds. It has also been previously used to determine the compounds present in *Ocimum*-based extracts [12-14]. The FTIR spectrum of our extracts were similar to that of the previous literature and were indicative that the leaf extracts of these herbs contained primary amines (3271 cm^{-1}), alkanes (2930-2973 cm^{-1}), aromatic rings (1590 cm^{-1}), and alkyl halides (520 cm^{-1}). Small shifts in the FTIR spectrum are associated with structural changes, which can be expected due to variations in the extraction techniques. It was interesting to note that although the same extraction technique was followed for the two herbs, the final product appeared different. *O. sanctum* extract was sticky in nature and exhibited increased peak intensities in FTIR analysis when compared to *O. gratissimum*. Higher peak intensities generally signify a greater amount of functional groups per unit volume. Small variations were also noted at 2970 cm^{-1}, and in the range of 600-400 cm^{-1}.

Previous literature has pointed out many potential pharmacological applications of *O. sanctum* and *O. gratissimum* extracts. Studies have also been conducted at the cellular level where *Ocimum* extracts exhibit cytotoxicity to cancer cells. Wihadmadyatami et al. [15] suggested that ethanol extract of *O. sanctum* induced an apoptosis on lung adenocarcinoma cell line at concentrations of 0.1 mg/mL and 0.2 mg/mL. Similarly, Huang et al. [16] suggested that the aqueous leaf extract of *O. gratissimum* induced cell apoptosis in a human hepatocellular carcinoma cell line at the concentration of 0.2 mg/mL. The increased cell apoptosis was due to increasing oxidative stress and loss of mitochondrial membrane potential. Phenolic phytochemical contents of herbs extract exhibited pro-oxidant activity, which can disrupt mitochondrial dehydrogenase activity of cancer cell and may also be the reason for increased cytotoxicity on cells. In addition, these two herbs are also known to produce wound healing activity. Somashekar et al. [7] indicated *O. sanctum* may be useful in abnormal healing and hypertrophic scar management because of flavonoid contents and antioxidant properties. Osuagwu et al. [8] implied that the methanolic extracts of *O. gratissimum* could enhance wound contraction and help in healing due to flavonoid contents [17]. Moreover, ethanol extracts from the leaves of *O. sanctum* and *O. gratissimum* have also shown anti-microbial activity. Kaypetch et al. [5] stated the essential oil of *O. sanctum* showed antibacterial effects in which *Streptococcus mutans* ATCC 25175 and *Streptococcus mutans* $KPSK_2$ strains were inhibited at minimum inhibitory concentrations (MIC) of 0.18 and 0.047 mg/mL and minimum bactericidal concentrations (MBC) of 0.377 and 0.095 mg/mL, respectively.

Moreover, this study was our first attempt to establish the cytotoxic effect of ethanol-based extracts on a normal cell line, using an in vitro cell culture model. Cytotoxicity is a cascade of molecular events of continuously obstructed macromolecular synthesis leading to unequivocal cellular, functional, and structural damage [18]. ISO guidelines recommends cytotoxicity testing as a primary step to determine the safety of health products for human usage. L929 mouse fibroblast cell line is a commonly used cell line for in vitro cytotoxic test of medical devices. It has the advantage of faster growth and good reproducibility with metabolic and genetic stability. It also has high sensitivity to toxic products and superior sensitivity when compared to primary human gingival fibroblasts [19]. MTT colorimetric assay was used to determine the mitochondrial function of the in-vitro cell. A color response is triggered when based on the reduction of the yellow tetrazolium salt to purple formazan crystal by metabolically active cells [9].

In our cytotoxic study, the overall results of extracts from the leaves of *O. sanctum* and *O. gratissimum* suggested that higher concentrations of herb extract might have toxic effects on the L929 cell line. In morphologic observations, the cells presented cell membrane breakage, cell shrinkage and lyses, and more than 50% growth inhibition was observed at concentrations of ≥0.93 mg/mL. On the other hand, cell growth without reduction and cell lysis occurred at concentrations ≤0.46 mg/mL, as shown by the MTT assay results at the lower concentrations of 0.23 and 0.46 mg/mL. There was a significant difference between the treatment and control groups. When compared between the test and control groups, L929 showed a significant difference at concentrations ≥0.93 mg/mL. The MTT assay also revealed the percentage of cell viability to be less than 70% when compared to the control group at concentrations ≥0.93 mg/mL. Only two concentrations, 0.46 and 0.23mg/mL, showed a greater percentage of cell viability with ethanol extract of *O. sanctum* at 77.81% and 101% respectively, and with *O. gratissimum* ethanol extract at 92.2% and 102.06%, respectively.

Limitations of this study included the use of an in-vitro test system, the results of which cannot be translated directly to clinical application. Furthermore, the results were based only on a fibroblast murine L929 cell line which is more sensitive than normal cell lines. Therefore, for the potential application of these herbal extracts for medicinal purposes, further studies on other murine or human cell lines from various tissues are suggested for which the values attained in this study may serve as a reference.

5. Conclusion

In this study, maceration, ethanol extraction, and spray drying techniques were followed to obtain powder extracts from the leaves of *O. sanctum* and *O. gratissimum*. FTIR results suggested that these extracts were composed of several bioactive components, mostly constituting of primary amines, alkanes, aromatic rings, and alkyl halides with small variations between the two herbs. The MTT assay revealed that both extracts presented no cytotoxicity at concentrations ≤0.46 mg/mL on murine L929 fibroblast cell line. However, at concentrations ≥0.93 mg/mL both extracts were toxic to the cell line and were significantly different when compared to the controls.

6. Acknowledgements

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7. References

[1] Joseph P, Hereman S. Paxton's botanical dictionary. 12nd ed. London: Bradbury Evans and Co.,;1868.
[2] Pattanayak P, Behera P, Das D, Panda SK. *Ocimum sanctum* Linn. A reservoir plant for therapeutic applications: an overview. Pharmacogn Rev. 2010;4(7):95-105.
[3] Bhavani T, Mohan RR, Mounica C, Nyamisha J, Krishna AG, Prabhavathi P, et al. Phytochemical screening & antimicrobial activity of *Ocimum gratissimum* review. J Pharma Phyto. 2019;8(2):76-79.
[4] Prabhu KS, Lobo R, Shirwaikar AA, Shirwaikar A. *Ocimum gratissimum*: a review of its chemical, pharmacological and ethnomedicinal properties. Open Complement Med J. 2009;1(1):1-15.
[5] Kaypetch R, Muadcheingka T, Tonput P. Antibacterial activity of *Ocimum sanctum* oil against Streptococcus mutans: in vitro. M Dent J. 2015;35(3):311-320.
[6] Talabi JY, Makanjuola SA. Proximate, phytochemical, and in vitro antimicrobial properties of dried leaves from *Ocimum gratissimum*. Prev Nutr Food Sci. 2017;22(3):191-194.
[7] Somashekar S, Saraswati U, Laxminarayana U. Evaluation of antioxidant and wound healing effects of alcoholic and aqueous extract of *Ocimum santum* Linn. in rats. Evid Based Complement Alternat Med. 2008;5(1):95-101.
[8] Osuagwu FC, Oladejo OW, Imosemi IO, Adewoyin BA, Aiku A, Ekpo OE, et al. Wound healing activities of methanolic extracts *Ocimum gratissimum* leaf in Wistar rats - a preliminary study. Afr J Med Med Sci. 2004;33(1):23-26.

[9] Archana D, Singh BK, Dutta J, Dutta PK. Chitosan-PVP-nano silver oxide wound dressing: in vitro and in vivo evaluation. Int J Biol Macromol. 2015;73:49-57.
[10] International Organization for Standardization. ISO 10993-5:2009(E). Biological evaluation of medical devices- part 5. Tests for in vitro cytotoxicity. 3rd ed. Geneva: ISO; 2009.
[11] Zhang QW, Lin LG, Ye WC. Techniques for extraction and isolation of natural products: a comprehensive review. Chin Med. 2018;13(20):1-26.
[12] Manogaran P, Muthukrishnan S. GC-MS and FT-IR analysis of phytocomponents in the methanolic extract of *Ocimum sanctum* (Tulsi). Int J Res and Analytical Rev. 2019;6(1):1023-1032.
[13] Pratheeba T, Ragavendran C, Natarajan D. Larvicidal, pupicidal and adulticidal potential *of Ocimum gratissimum* plant leaf extracts against filariasis inducing vector. Int J Mosquito Res. 2015;2:1-8.
[14] Raju RS, Sakuntala P, Jaleeli KA. FT-IR sectroscopy analysis of *Ocimum gratissimum* leaves. Int J Emerging Technologies in Engineering Res. 2017;5(4):131-133.
[15] Wihadmadyatami H, Karnati S, Hening P, Tjahjono Y, Rizal, Maharjanti F, et al. Ethanolic extract *Ocimum sanctum* Linn. induces an apoptosis in human lung adenocarcinoma (A549) cells. Heliyon. 2019;5(8):02772.
[16] Huang CC, Hwang JM, Tsai JH, Chen JH, Lin H, Lin GJ, et al. Aqueous *Ocimum gratissimum* extract induces cell apoptosis in human hepatocellular carcinoma cells. Int J Med Sci. 2020;17(3):338-346.
[17] Ielpo MT, Basile A, Miranda R, Moscatiello V, Nappo C, Sorbo S, et al. Immunopharmacological properties of flavonoids. Fitoterapia. 2000:101-109.
[18] Ozdemir KG, Yilmaz H, Yilmaz S. In vitro evaluation of cytotoxicity of soft lining materials on L929 cells by MTT assay. J Biomed Mater Res B Appl Biomater. 2009;90(1):82-86.
[19] Thonemann B, Schmalz G, Hiller KA, Schweikl H. Responses of L929 mouse fibroblasts, primary and immortalized bovine dental papilla-derived cell lines to dental resin components. Dent Mater. 2002;18:318-323.