

An ethanol extract of Senduduk fruit (*Melastoma malabathricum* L) inhibits the expression of vascular endothelial growth factor and tumour necrosis factor alpha in HeLa cells

Deri Edianto¹, Aznan Lelo², Syafruddin Ilyas³, Marline Nainggolan⁴

¹Department of Obstetrics and Gynaecology, ²Department of Pharmacology and Therapeutics; Faculty of Medicine Universitas Sumatera Utara, ³Faculty of Mathematics and Natural Science, ⁴Faculty of Pharmacy; Universitas Sumatera Utara, Indonesia

ABSTRACT

Aim Senduduk fruit (*Melastoma malabathricum* L) is native to Indomalaya and is believed to possess anticancer activity. This study investigated antiangiogenic and anti-inflammation effects of an ethanolic extract of Senduduk fruit (EESF) on HeLa cells.

Methods Cytotoxicity was assayed in HeLa cell cultures exposed to a concentration series of 500–7.8 µg/mL of EESF. IC₅₀ was determined with a methylthiazol-tetrazolium (MTT) cell viability assay. Antiangiogenic and anti-inflammation activity was evaluated by an immunocytochemical assay of vascular endothelial growth factor (VEGF) and tumour necrosis factor alpha (TNF-α) expression in HeLa cells cultured with 1× or 2× IC₅₀ or as a control without EESF.

Results IC₅₀ of the EESF was 956 µg/mL. The intensity of VEGF staining indicated moderate expression in HeLa cells in response to IC₅₀, weak expression in response to 2×IC₅₀, and strong expression in the absence of the EESF. While the intensity of TNF-α staining indicated moderate expression in HeLa cells in response to IC₅₀ and to the absence of EESF, and weak expression in response to 2× IC₅₀.

Conclusion Senduduk fruit extract inhibited VEGF and TNF-α expression in HeLa cells in a concentration-dependent manner.

Key words: ethanol extract, HeLa cell, *Melastoma malabathricum* L fruit, TNF-α, VEGF

Corresponding author:

Deri Edianto
Department of Obstetrics and
Gynaecology, Faculty of Medicine,
Universitas Sumatera Utara
Jalan Suka Menang no 18, 20146 Medan,
North Sumatera, Indonesia
Phone: +62 81 1639 232;
E-mail: deri.edianto@usu.ac.id
ORCID ID: <https://orcid.org/0000-0002-1459-9523>

Original submission:

15 April 2020;

Revised submission:

08 June 2020;

Accepted:

22 June 2020

doi: 10.17392/1182-20

Med Glas (Zenica) 2020; 17(2): 414-419

INTRODUCTION

Noncommunicable diseases are responsible for the majority of deaths worldwide, and cancer is a leading cause (1). The reasons are complex, but reflect both aging and growth of the population (2,3). According to Globocan 2018, cervical cancer is the fourth most frequently diagnosed cancer and the fourth leading cause of cancer deaths in women, with an estimated 570,000 cases and 311,000 deaths worldwide (4). Approximately 80% of new cases occur in countries without effective screening programs (5). Cervical cancer is generally treated by surgery or radiation. Both have side effects and recurrence may occur (6,7). Novel pharmacological breakthroughs are needed, such as natural extracts that are effective with minimal toxicity (8).

The carcinogenesis of cervical cancer is a multistep process that causes a progressive transformation of normal cells into cancer cells with disease-specific characteristics (9). Sustained proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activation of invasive and metastatic behaviour are key properties of cancer cells, named cancer hallmarks (10). Angiogenesis, the initiation and growth of new blood vessels, ensuring the supply of oxygen and nutrients required to maintain tumour growth and support metastasis (11). Vascular endothelial growth factor (VEGF) stimulates the formation of new blood vessels (12).

In the early 19th century, it was perceived that cancer was related to inflammation. Recent efforts have shed new light on molecular and cellular pathways that connect inflammation and cancer. Inflammation is a target for pharmacologic intervention (13). One of the main chemical mediators involved in cancer-related inflammation is TNF- α (14).

Treatment failure and the high systemic toxicity of conventional cancer therapies have prompted a search for a novel agent with anticancer activity and low toxicity (15). Polyphenols, alkaloids, nitrogen compounds, and carotenoids are bioactive phytochemicals that can interfere with many signaling pathways that regulate cell proliferation and tumour initiation and growth (16). Vegetables, grains, fruits, and other plant products are rich in phytochemicals, and diets including a variety of fruits and vegetables may lower the risk

of cancer (17). The chemopreventive anticancer activities of more than 1000 phytochemicals have been investigated (18). Bioflavonoids are plant secondary metabolites with potential human health benefits. They are relatively abundant in plant foods and are nontoxic (19). Polyphenols are a class of flavonoids that have low bioavailability but are rapidly metabolized (20). The anticancer activity of polyphenols has been investigated, and a few trials in cervical cancer have primarily involved green tea and curcumin (16).

This study investigated the anticervical cancer activity of a *Melastoma* (Senduduk) fruit extract. Ripe Senduduk fruits are purplish black, have a sweet and sharp taste. The *Melastoma* plants are native to Sumatera, Borneo, and the Malay Peninsula (21).

Melastoma malabathricum L (Figure 1) is an ornamental plant with white or pink-purple flowers, included in the *Melastomataceae* family, which spread in Asia especially in Southeast Asia. Some regions are approved under different names. Traditionally, it has been known to have many medicinal benefits such as antibacterial, antiviral, anti-inflammatory, anti-parasite, antioxidant, anticancer, anticoagulant, platelet activating factor inhibitor, antiulcer, antidiarrhea, antivenom, fever and wound healing (22).



Figure 1. Senduduk Plant (*Melastoma malabathricum* L) (Edianto D, 2019)

Melastoma genus in Southeast Asia consists of 22 species, 2 subspecies, and 3 varieties. These varieties are based on the colour of flower petals, which are pink, white, and purple. Pink flower petal varieties are often found in Indonesia and Malaysia. The number of flower petals is five. The fruit colour is deep purple, soft, and contains many fine orange seeds (21).

Senduduk fruit (*Melastoma malabathricum* L) is known to contain anthocyanins such as cyanidin di-hexoside, cyanidin hexoside, delphinidin hexoside, and pelargonidin, but the use of active ingredients from this fruit has not been reported (22-25).

MATERIAL AND METHODS

Materials and study design

This study was conducted at the Faculty of Pharmacy, Universitas Sumatera Utara, Medan and Department Parasitology, Faculty of Medicine Universitas Gadjah Mada, Yogyakarta, Indonesia in the period April - May 2019.

The approval for the investigation was obtained from the Ethics Committee of the Health Research Ethical Committee, Faculty of Medicine Universitas Sumatera Utara Indonesia.

Whole ripe of Senduduk fruit (*Melastoma malabathricum* L) collected from abandoned, vacant land in Pasar Rawa Village, Gebang District, Langkat Regency, North Sumatera Province, Indonesia was used in this study. The extract was made in the phytochemical laboratory of the Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia. Prior to the extraction, the fruit was separated from its stalk, squeezed to obtain the pulp, and dried in a dehydrator at 40°C until it was brittle. The ethanol extract of Senduduk fruit (EESF) was made by maceration of the pulp in 80% ethanol. The extracts were put in the dark red glass bottle and kept in freeze on refrigerator before the use. The flavonoid components of the extract were evaluated by spectrophotometry (PT Saraswanti Indo Gene-tech, Bogor, Indonesia).

Methods

HeLa cells. HeLa cells were obtained from the Laboratory of Parasitology at the Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. The cells were cultured in Roswell Park Memorial Institute medium (RPMI) (Sigma Aldrich, USA) containing 10% fetal bovine serum, 0.5% fungizone (Amphotericin B) and 2% penicillin-streptomycin and incubated in Heraeus Hera Cell incubator (Thermo Fischer Scientific, Waltham, MA, USA) at 37°C and 5% CO₂. They were harvested with trypsin-EDTA (Sigma Aldrich, USA) after single layer cells

grow confluent at least 80%, which were evaluated using inverted light microscope (Olympus CKX41, Japan).

Cytotoxicity assay. HeLa cells were transferred to 96-well plates (10⁴ cells/well) in 100 µL culture mediums containing 500, 250, 125, 62.5, 31.25, 15.625, or 7.8125 µg/mL of senduduk extract with six replicate wells for each concentration. The control cultures included culture medium without cell and cell control with no added extract. The culture medium was discarded after overnight incubation and replaced with 100 µL methylthiazol-tetrazolium (MTT – Sigma Aldrich, USA) solution and incubated for 2 hours. The MTT reaction was stopped with 10% sodium dodecyl sulfate (SDS) in 0.01 N HCl (Sigma Aldrich, USA) after formation of formazan, the plates were kept overnight in the dark at room temperature, and the tetrazolium absorbance was measured at 595 nm using a culture micro plate reader (Bio-Rad Bechmark, USA). Cell viability was calculated as

$$\% \text{ Cell viability} = \frac{(C - B)}{(A - B)} \times 100\%$$

where A = cell control (cells + media), B = media control, C = sample + media + cells. IC₅₀ was calculated with Microsoft Excel.

Vascular endothelial growth factor (VEGF) and tumour necrosis factor alpha (TNF-α) expression. HeLa cells were transferred to 24-well plates at 100,000 cells/well in RPMI with coverslip inside and incubated overnight. The EESF was added at 1× or 2× IC₅₀ to all but not the control wells and reincubated overnight for each marker. VEGF and TNF-α expression was assayed at the coverslip after overnight reincubation with a primary anti-VEGF C-1, sc-7269 antibody (Santa Cruz Biotechnology Inc, USA) and anti-TNF-α ab6671 antibody (Abcam, USA), and a secondary antibody reagent universal detection kit (Paramount BGD-0100, Bio-Gear, USA). VEGF and TNF-α expression was assessed at the anatomical pathology laboratory using light microscope by two pathologists and was graded as strong, moderate, or weak by the intensity of brown staining.

RESULTS

Spectrophotometry revealed that the EESF contained anthocyanin, quercetin, flavone, katechin, and other components. EESF contains 115.70 mg/kg anthocyanin, and the derivatives are delphinidin and cyanidin 3-glucoside.

In this research, HeLa cells were cultured for 24 hours in RPMI media that contain EESF in various concentrations. Cell viability was measured using the MTT assay. The highest concentration starts with 500 $\mu\text{g/mL}$, half of it was diluted at the next concentration to get IC_{50} . And it turns out that the highest dose only inhibits the viability of HeLa cells by around 70%. The results of the MTT assay of EESF cytotoxicity at 500–7.8125 $\mu\text{g/mL}$ were expressed as a percentage of viable HeLa cells (Figure 2). IC_{50} of the EESF calculated by extrapolation using Excel programme was 956 $\mu\text{g/mL}$.

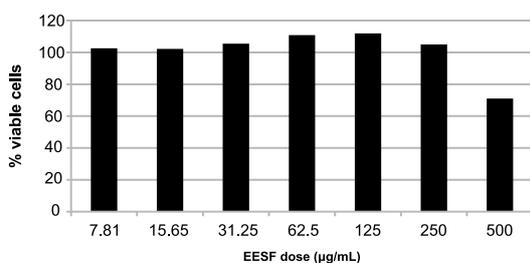


Figure 2. Methylthiazol-tetrazolium (MTT) assay of cytotoxicity as a percentage of viable HeLa cells; EESF, ethanol extract of senduduk fruit

The presented research assessed anticancer benefits, especially the anti-angiogenesis and anti-inflammation effects. Because IC_{50} was high, VEGF and TNF- α expression were evaluated in HeLa cells treated with 1 \times and 2 \times IC_{50} level.

By giving these plant extracts to HeLa cell cultures with concentrations of 1X and 2X IC_{50} , respectively, and as a control without extracting, through immunocytochemical examination VEGF and TNF- α expression were assessed. Two pathologist gave the same examination results, and there was no need for Kappa test. HeLa cells without administration of the extract express strong VEGF, and their expression decreased with increasing concentration of the extract.

VEGF expression in HeLa cell control, EESF 1 \times IC_{50} , and 2 \times IC_{50} cultures is shown in Figure 3. VEGF expression was assessed by the intensity of brown staining in HeLa cells. The intensity was strong in cells from control cultures without EESF, moderate in cultures with 1 \times EESF IC_{50} , and weak in cultures with 2 \times EESF IC_{50} .

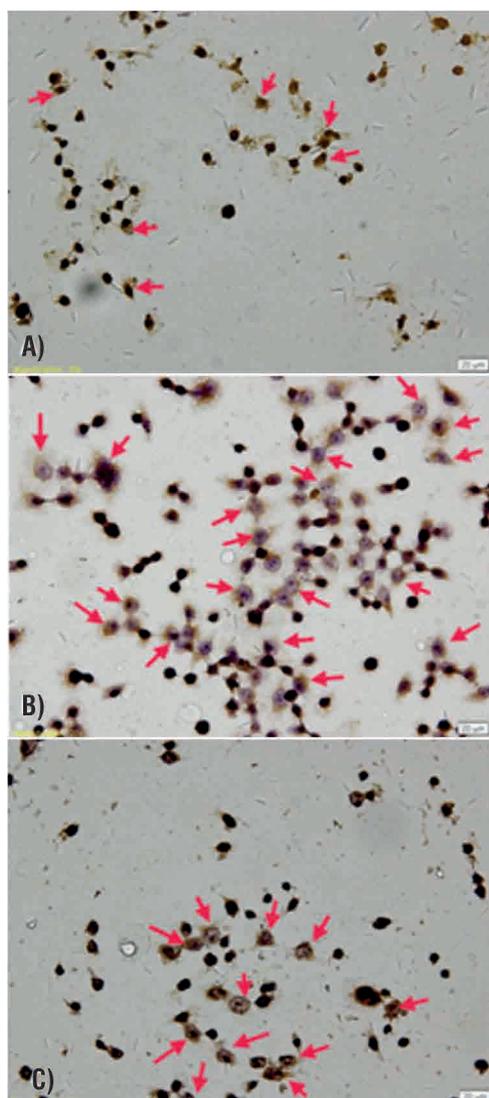


Figure 3. Vascular endothelial growth factor (VEGF) expression in HeLa cells. A) in control cultures, B) cultured with 1 \times EESF IC_{50} , C) cultured with 2 \times EESF IC_{50}

Without the administration of extracts, HeLa cells only showed the same moderate expression of TNF- α as if given extracts with a concentration of 1X IC_{50} . Increasing the dose of extract turned down its expression.

TNF- α expression in HeLa cell control, EESF 1 \times IC_{50} , and 2 \times IC_{50} cultures is shown in Figure 4.

The intensity was moderate in cells control cultures without EESF and also with 1 \times EESF IC_{50} , and weak in cultures with 2 \times EESF IC_{50} .

DISCUSSION

IC_{50} of 956 $\mu\text{g/mL}$ was estimated in HeLa cell cultures containing EESF at concentrations ranging from 500 $\mu\text{g/mL}$ to 7.8125 $\mu\text{g/mL}$ obtained

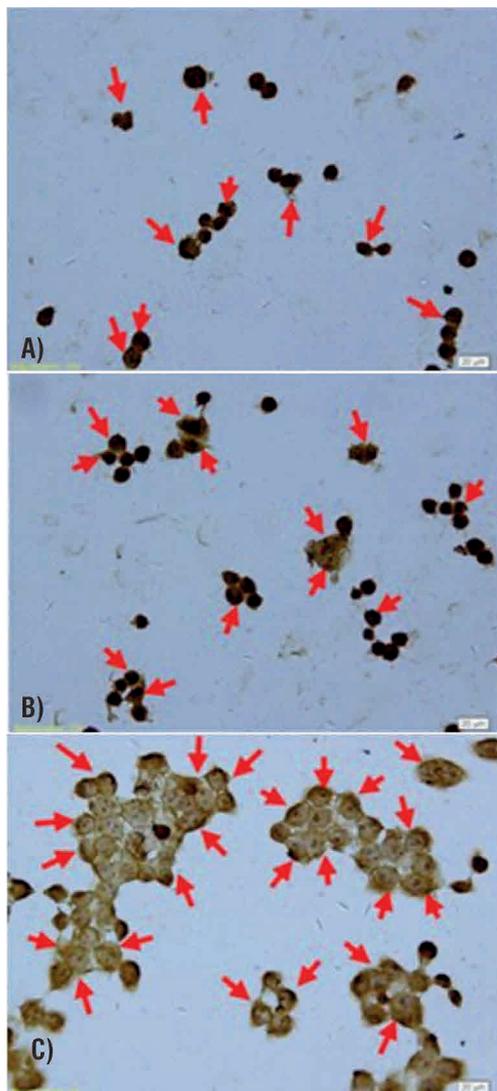


Figure 4. Tumour necrosis Factor- α (TNF- α) expression in HeLa cells. A) in control cultures, B) cultured with 1x ethanol extract of senduduk fruit (EESF IC₅₀), C) cultured with 2x EESF IC₅₀

by serial dilution that reduced the dose by half at each step. It was much than $1.781 \pm 1.2 \mu\text{g/mL}$ reported by Alnajjar et al. (26) for the inhibition of peripheral blood mononuclear-cell proliferation. Roslen et al. (21) found that IC₅₀ of a Senduduk fruit extract for *Artemia salina* brine shrimp larvae was 89.947 ppm.

Flavonoids, tannin, anthocyanin, and phenol compounds are among the bioactive components of Senduduk fruit with pharmacologic benefits (22). Anthocyanins are water-soluble plant pigments that are responsible for the red, blue, purple, and black colour of fruits. They are ubiquitous in plants and are the most abundant flavonoid constituent of fruits (27). The study results through spectrophotometry examination revealed that the Senduduk fru-

it extract contains delphinidin, cyanidin 3-glucoside, quercetin, flavon, epicatechin and luteolin, and it is in line with previous reports that plant extracts containing phenol compounds like flavonoids and anthocyanin have anticancer activity (22). Amatori et al. reported that extracts of the alba variety of strawberries that contained phenol compounds inhibited the growth of A17 breast cancer cells compared with normal control cells (28). The extract used in this study contained constituents other than anthocyanin, which may contribute to its activity.

Our study showed that the greater EESF had stronger effect on VEGF expression. Samad et al. reported that a methanol extract of Senduduk fruit had antiproliferative activity against HeLa cells but did not inhibit the proliferation of 3T3 cells, indicating its lack of toxicity to normal cells; the high IC₅₀ level observed will most likely to be harmless to normal cells (29).

Angiogenesis is a hallmark cancer characteristic and is needed for continuing tumour development (10,30). Continuous blood vessels growth is required to supply the oxygen and nutrient support for increased growth of more than 1–2 mm³ in tumour size. VEGF stimulates angiogenesis and VEGF expression is triggered by hypoxia (31,32).

Inflammation is one of the complex biological responses to the damages caused either by injury or microbial infection. The role of inflammation in tumorigenesis is now widely accepted. In many cases, chronic inflammation in the micro-environment is essential for the initiation and progression the cancers (32). TNF- α is involved in maintenance of the inflammation and host defence. However, there is a ‘dark side’ to this powerful cytokine in the pathological process such as malignant disease (11).

A high dose of the EESF was required to inhibit VEGF and TNF- α expression in this *in vitro* study. Because the oral bioavailability of flavonoids is low, it will be difficult to achieve this high dose in an *in-vivo* studies. In conclusion, ethanol extract of Senduduk fruits reduced VEGF and TNF- α expression in HeLa cells in a dose-dependent manner.

FUNDING

No specific funding was received for this study

TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.

REFERENCES

- World Health Organization. Global health observatory. Geneva: World Health Organization 2018.
- Omran AR. The epidemiologic transition . A theory of the epidemiology of population change. *Milbank Mem Fund Q* 1971; 49:509-38.
- Gersten O, Wilmoth JR. The cancer transition in Japan since 1951. *Demogr Res* 2002; 7:271-306.
- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistic: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; 68:394-424.
- Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tiu-lent, Jemal A. Global cancer statistic, 2012. *CA Cancer J Clin* 2015; 65:65-87.
- Marnitz S, Kohler C. Advanced Cervical Cancer. In: Ayhan A, Reed N, Gultekin M, Dursun P, eds. *Textbook of Gynaecological Oncology*. 2nd ed. Ankara: Gunes Publishing, 2012:361-71.
- Carter J, Philp S, O'connell R. A 5-year review of gynecological oncology patients managed by fast tract surgery program. *Int J Clin Med* 2014 2014; 5:36-41.
- Xu H, Zhou Z, Dong J, Lei M. Suppression of cervical cancer cell survival by ursolic acid extracted from *Catalpa bungei* leaves. *Pharmacogn Mag* 2018; 14:425-31.
- Wang RA, Li QL, Li ZS, Zheng PJ, Zhang HZ, Huang XF, Chi SM, Yang AG, Cui R. Apoptosis drives cancer cells proliferate and metastasize. *J Cell Mol Med* 2013; 17:2015-11.
- Hanahan D, Weinberg RA, Hallmarks of cancer: the next generation. *Cell* 2011; 144:646-74.
- Zhong H, Bowen JP. Antiangiogenesis drug design: multiple pathways targeting tumor vasculature. *Curr Med Chem* 2006; 13:849-62.
- Kukreja I, Kapoor P, Deshmukh R, Kulkarni V. VEGF and CD34: A correlation between tumor angiogenesis and microvessel density – an immunohistochemical study. *J Oral Maxillofac Pathol* 2013; 17:367-73.
- Mantovani A. Tumor-associated macrophage in cancer-related inflammation. *Immunotherapy* 2011; 3:21-2.
- Balkwill F. TNF- α in promotion and progression of cancer. *Cancer Metastasis Rev* 2006; 25:409-16.
- Fimognari C, Lenzi M, Hrelia P. Chemoprevention of cancer by isothiocyanates and anthocyanins: mechanisms of action and structure activity relationship. *Curr Med Chem* 2008; 15:440-7.
- Moga MA, Dimienescu OG, Arvatescu CA, Mironescu A, Dracea L, Ples L. The role of natural polyphenols in the prevention and treatment of cervical cancer – an overview. *Molecules* 2016; 21:1-32.
- Batra P, Sharma AK. Anti-cancer potential of flavonoids: recent trends and future perspectives. *3 Biotech* 2013; 3:439-59.
- Karikas GA. Chemoprevention molecular and biochemical mechanisms involved in cancer control and management. *Health Sci J* 2011; 5:149-56.
- Hui C, Bin Y, Xiaoping Y, Long Y, Chunye C, Mantian M. Anticancer activities of an anthocyanin-rich extract from black rice against breast cancer cells *in vitro* and *in vivo*. *Nutr Cancer* 2010; 62:1128-36.
- Sak K. Cytotoxicity of dietary flavonoids on different human cancer types. *Pharmacogn Rev* 2014; 8:122-46.
- Roslen NA, Alewi NAM, Ah reamada H, Rasad MSBA. Cytotoxicity screening of *Melastoma malabathricum* extracts on human breast cancer cell line *in vitro*. *Asian Pac J Trop Biomed* 2014; 4:545-48.
- Aslam MS, Ahmad MS, Ahmad MA. An up-to-date review on phytochemical constituents and pharmacological activities of *Melastoma malabathricum*. *Int J Pharm Sci Res* 2017; 8:76-91.
- Anuar N, Adnan AFM, Saat N, Taha RM. Optimization of extraction by using response surface methodology, purification and identification of anthocyanin pigments in *Melastoma malabathricum* fruit. *Sci World J* 2013; 1-10.
- Nath D, Choudhury MD, Muzamber PB, Mitra A. Phytochemical profiling of *Melastoma malabathricum linnaeus (Melastomaceae)*: an ethnomedicinally important plant of eastern Himalaya. *Pleione* 2014; 8:478-85.
- Serna DMO, Martinez JHI. Phenolic and polyphenolic from *Melastoma malabathricum* species. *Molecules* 2015:17818-47.
- Alnajjar ZAA, Abdullah MA, Ali HM, Alshawsh MA, Hadi AAH. Acute toxicity evaluation, antibacterial, antioxidant and immunomodulatory effects of *Melastoma malabathricum*. *Molecules* 2012; 17:3547-59.
- Khoo HE, Azlan A, Tang ST, Lim SM. Anthocyanidins and anthocyanins: colored pigment as food pharmaceutical ingredients, and the potential health benefits. *Food Nutr Res* 2017; 61:1-21.
- Amatori S, Mazzoni L, Alvarez-Suarez JM, Giamperri F, Gasparrini M, Forbes-Hernandez TY, Afrin S, Prorenzano AE, Persico G, Mezzeti B, Augusto A, Faneli M, Battino M. Polyphenol-rich strawberry extract (PRSE) shows *in vitro* and *in vivo* biological activity against invasive breast cancer cells. *Sci Rep* 2016; 6:1-14.
- Samad NA, Nik NNS, Kamal M, Yahya N, Aziz MY, Zain NNM, Yusoff NAM, Lim V. Ethnobotanical, phytochemical and pharmacological aspects of *Melastoma* sp. *Mal J Med Health Sci* 2018; 14:153-63.
- Mirossay L, Varinska L, Mojzis J. Antiangiogenic effect of flavonoids and
- Zetter BR. Angiogenesis and tumor metastasis. *Annu Rev Med* 1998; 49:407-24.
- Chung SS, Okobi Q, Adekoya D, Atefi M, Clarke O, Dutta P, Vadgama JV. Proinflammatory cytokines IL-6 and TNF- α increased telomerase activity through NF- κ B/STAT1/STAT3 activation and witherferin a inhibited the signaling in colorectal cancer cells. *Mediat Inflamm* 2017; 2017-5958429.