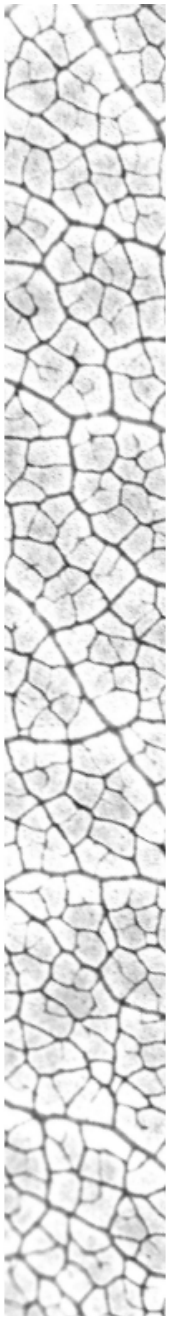

EurAsian Journal of BioSciences
Eurasia J Biosci 13, 49-55 (2019)

Investigation of the biological activities of Phu Quoc Sim fruits *Rhodomyrtus tomentosa* (aiton) hassk

Vo Thanh Sang [1*], Ngo Dai Hung [2], Le Phuong Uyen [1], Ngo Dai Nghiep [3], Kim Se-Kwon [4]**

[1] NTT Institute of Hi-Technology, Nguyen Tat Thanh University, Ho Chi Minh City, VIETNAM
[2] Faculty of Natural Sciences, Thu Dau Mot University, Binh Duong province, VIETNAM
[3] Department of Biochemistry, Faculty of Biology and Biotechnology, University of Science, Vietnam National University, Ho Chi Minh City, VIETNAM
[4] Department of Marine Life Science, College of Ocean Science and Technology, Korea Maritime and Ocean University, Busan 606-791, SOUTH KOREA
*Corresponding author: vtsang@ntt.edu.vn
**Corresponding author: hungnd@tdmu.edu.vn

Abstract

Background: *Rhodomyrtus tomentosa* (Aiton) Hassk., locally known as Sim, is a flowering plant belonging to the family Myrtacea. It have been used in traditional Vietnamese medicine for a long time for treatment of diarrhoea, dysentery, gynaecopathy, stomachache, and wound healing. In this study, the potential bioactivities of ethanol extract of Sim fruits due to scavenging free radicals, suppressing pro-inflammatory mediator production, and inhibiting histamine release were investigated in vitro.

Material and Methods: Sim fruits were obtained from from Duong Dong Town, Phu Quoc district, Kien Giang province, Viet Nam. The crude extract was prepared with Sim fruits in ethanol 80%. The free radical scavenging activity was conducted via DPPH and ABTS assay. Nitric oxide (NO) production was measured by colorimetric assay and inducible nitric oxide synthase (iNOS) expression was conducted by Western blot. Histamine release was measured by spectrofluorometric assay.

Results: Ethanol extract Sim fruits (EESF) was found to be able to scavenge DPPH and $ABTS^{+}$ radicals with IC50 values of 94 ± 12 and 83 ± 9 µg/ml, respectively. Moreover, NO production and iNOS protein expression were suppressed by EESF treatment at a dose-dependent manner in lipopolysaccharide-stimulated RAW 264.7 cells. In addition, EESF was effective in reducing histamine release from calcium ionophore-activated RBL-2H3 mast cells.

Conclusion: These results indicated the potential health benefit effects of Sim fruit due to its biological activities. These evidences supported it as a promising biomaterial for alternative medicine.

Keywords: Sim fruit, *Rhodomyrtus tomentosa*, DPPH, histamine, iNOS

Sang VT, Hung ND, Uyen LP, Nghiep ND, Se-Kwon K (2019) Investigation of the biological activities of Phu Quoc Sim fruits *Rhodomyrtus tomentosa* (aiton) hassk. Eurasia J Biosci 13: 49-55.

INTRODUCTION

Natural products including the herbal remedy, medicinal plants, functional foods, and their constituents have been applied for the prevention and/or treatment of numerous diseases (Kim et al. 2018). The capacity of some natural product-derived foods to reduce the risk of chronic diseases has been associated, at least in part, to the occurrence of secondary metabolites that have been shown to exert a wide range of biological activities such as anti-viral, anti-oxidant, anti-inflammatory, anti-allergic, anti-cancer, anti-diabetes, anti-obesity, anti-hypertensive, and immuno-enhancing activities (David et al. 2014, Dias et al. 2012, Vo and Kim 2013). Thus, customers have got more interesting forward to natural product-derived foods nowadays.

Plants of the *Myrtaceae* family are known to contain a rich source of structurally diverse and biologically active metabolites, thus considering as a potential resource for exploring novel therapeutic agents (Hazrulrizawati et al. 2017). *Rhodomyrtus tomentosa*, locally known as Sim, is a flowering plant belonging to the family Myrtaceae, native to Southern and Southeastern Asia (Lim 2012). Parts of this plant have been used in traditional Vietnamese, Chinese and Malay medicine for a long time. In particular, the fruits have been used to treat diarrhoea, dysentery, gynaecopathy, stomachache, and wound healing (Dachriyanus et al. 2002). The chemical constituents of *R. tomentosa* have been reported to include triterpenes, steroids, and phenolic compounds (Lai et al. 2015). Although *R. tomentosa* has been reported recently (Cui et al. 2013,

Received: October 2018
Accepted: December 2018
Printed: February 2019

Geetha et al. 2010, Le et al. 2018, Saising et al. 2008), the further studies on their biological activities are still limited. Thus, the main purpose of this contribution is to investigate the biological activities of Sim fruit *R. tomentosa* due to due to scavenging free radicals, suppressing pro-inflammatory mediator production, and inhibiting histamine release in vitro.

MATERIALS AND METHODS

Materials

Sim fruits were purchased from Duong Dong Town, Phu Quoc district, Kien Giang province, Vietnam. Solvent was purchased from Xilong (China). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Extraction

Sim fruits were air-dried under shade and powdered using a grinder. The powder was soaked with ethanol 80% under the extract conditions of ratio (1/4, w/v), time (4 h) and temperature (60 °C). The crude extract was dissolved in DMSO for further investigation.

1,1-Diphenyl-2-picryl-hydrazyl assay

The antioxidant activity of EESF was determined by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay as described by Vo et al. (2018b). Briefly, 100 µl of EESF (500 µg/ml) was mixed with 100 µl of DPPH solution and incubated in the dark at room temperature for 30 min. The absorbance of the mixture was then measured at 490 nm by using Genova Nano (Jenway, UK). The ability of the sample to scavenge DPPH radical was determined from:

$$DPPH\ scavenging\ effect = [(OD_{control} - OD_{sample})/OD_{control}]\ x\ 100\%$$

2,2-Azinobis-3-Ethyl benzothiazoline-6-sulfonic acid (ABTS) assay

This assay was performed as described by Vo et al. (2017). Briefly, the radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and leaving the mixture for 4-16 h until the reaction was completed and the absorbance was stable. The $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.05 at 734 nm for measurement. The photometric assay was conducted on 0.9 ml $ABTS^+$ solution and 0.1 ml of EESF (500 µg/ml), mixing for 45 sec. Measurement was taken immediately at 734 nm after 15 min. Ascorbic acid was used as a positive control. The antioxidative effect was calculated by determining the decrease in the absorbance at different concentrations by using the following equation:

$$ABTS\ scavenging\ effect = [(OD_{control} - OD_{sample})/OD_{control}]\ x\ 100\%$$

Cell Culture

The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C using Dulbecco's modified eagle medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES buffer, 100 U/ml of penicillin G, and 100 mg/ml of streptomycin. The cells were maintained via two times passage per week, and cells were utilized for experimentation at a density of 70–80%.

Cell Viability

The viability levels of the cells were determined by MTT assay. In brief, cells ($1x10^5$ cells/ml) were incubated with the indicated concentrations of EESF (100, 200, and 500 µg/ml) for 24 h. The medium was removed, and the cells were incubated with a solution of 1 mg/ml MTT for 4 h. Finally, the supernatant was removed, and DMSO was added to solubilize the formed formazan salt. The amount of formazan salt was determined by measuring the absorbance at 540 nm using a microplate reader (GENios® Tecan Austria GmbH, Austria).

NO Production Assay

NO level in the culture supernatant was measured by the Griess reaction as described earlier (Vo et al., 2012b). In brief, RAW 264.7 cells were plated at $1x10^5$ cells/ml in 24-well culture plates, and treated with various concentrations of EESF (100, 200, and 500 µg/ml) for 12 h prior to stimulation of LPS (1 µg/ml, final concentration) for 12 h. Aliquots (50 µl) of the supernatants were incubated with 50 µl of Griess reagent for 15 min. This was followed by measurement of absorbance values at 540 nm by using Genova Nano (Jenway, UK). The nitrite concentrations were calculated with reference to a standard curve of sodium nitrite generated by known concentrations.

Histamine Release Assay

RBL-2H3 cells were seeded into 24-well plates (2×10^5 cells/ml). Cells were treated with different concentrations of EESF (100, 200, and 500 µg/ml) for 12 h. The treated cells were washed by Tyrode buffer and stimulated with calcium ionophore A23187 (1 µM, final concentration) at 37 °C for 30 min. Histamine release in the supernatants was determined as previously described (Vo et al., 2011). Histamine release levels were calculated as a percentage compared to control: Release ratio (%) = (T – B)/(C – B) × 100, where B is the group without stimulation as well as sample treatment, C is the stimulated group without treatment of the tested sample, and T is the stimulated group with presence of the tested sample.

Western Blot Analysis

RAW 264.7 cells were treated with different concentrations of EESF (100, 200, and 500 µg/ml) for 12 h before stimulation of LPS for 12 h (1 µg/ml, final concentration). The cells were lysed in RIPA lysis buffer. The detection of protein was performed as described by Vo et al. (2014).

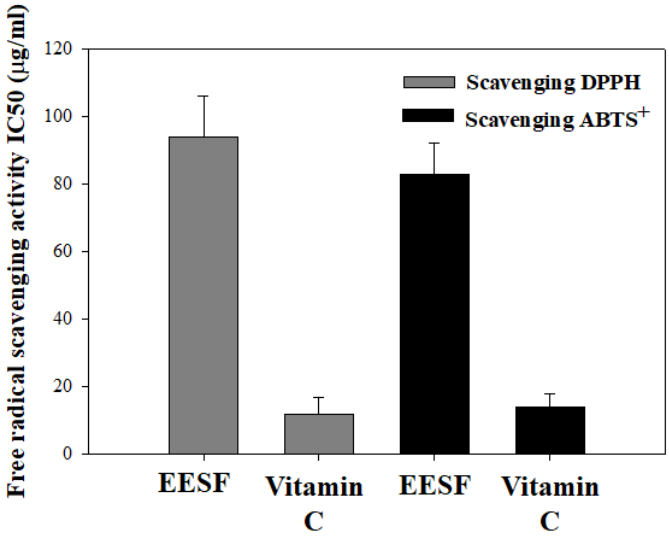

Fig. 1. The DPPH ana ABTS$^+$ radical scavenging activity of EESF and vitamin C. Each determination was made in triplicate, and the data are expressed as means ± SD. EESF: Ethanol extract Sim fruit

Statistical Analysis

Values were expressed as the mean ± SD. Statistical analysis was done using Student's *t-test*. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The Free Radical Scavenging Activity of EESF

Oxidative stress is an important risk factor in the pathogenesis of numerous chronic diseases. Free radicals are recognized as agents involved in the pathogenesis of sicknesses such as allergy, inflammation, diabetes, Parkinson's and Alzheimer's diseases, cancers as well as atherosclerosis (Kanwar et al. 2009). Meanwhile, an antioxidant is known to be substance that can trap the free radicals. Herbal plants considered as good antioxidant since ancient times (Wu et al. 2011). In this study, the antioxidant activity of EESF was investigated via measuring DPPH and ABTS$^+$ radical scavenging ability. **Fig. 1** showed that EESF was effective in trapping DPPH and ABTS$^+$ free radicals with IC50 values of 94 ± 12 and 83 ± 9 µg/ml, respectively. Vitamin C, a positive control, scavenged DPPH and ABTS$^+$ free radicals with IC50 values of 12 ± 5 and 14 ± 4 µg/ml, respectively. According to Ghasemzadeh et al. (2012), various tropical plants were investigated for antioxidant activity via scavenging DPPH radical. The tropical plant extracts including cabbage, green chilli, red chilli, carrot, lemon grass, and turmeric were showed to scavenge DPPH radical with IC50 values of 480 ± 12, 575 ± 10, 376 ± 14, 405 ± 17, 448 ± 16, 600 ± 15, respectively. It indicated that EESF possesses stronger DPPH scavenging activity than that of these tropical plant extracts.

The Suppressive Effect of EESF on NO Production and iNOS Protein Expression

Inflammation is the response of host to various stimuli including physical damage, ultra violet irradiation, microbial invasion, and immune reactions (Gautam and Jachak 2009). The inflammatory response is associated with the production of a large range of pro-inflammatory mediators such cytokine, NO, and PGE_2 that initiate the inflammatory response (Gordon 1998). Thus, down-regulation of mediator production during inflammatory response is necessary for reducing inflammatory symptom. As show in **Fig. 2A**, lipopolysaccharide (LPS) stimulation causes the production of NO from RAW 264.7 cells (34 µM). Conversely, EESF treatment leaded to decrease NO production in a dose-dependent manner. At the concentration of 500 µg/ml, NO level significantly reduced upon 14 µM. Notably, the inhibitory effect of EESF on NO production was not due to cytotoxic effect (**Fig. 2B**). Indeed, the iNOS protein is responsible for the production of NO from the oxidation of L–arginine (McAdam et al. 2012). The increased iNOS protein expression leads to the increase in NO in many chronic inflammatory diseases (Grabowski et al. 1997, McInnes et al. 1996). Herein, the suppressive effect of EESF on iNOS protein expression was also tested in LPS-stimulated RAW 264.7 cells. As shown in the **Fig. 2C**, EESF induced a dose-dependent suppression on protein expression of iNOS as compared to the control group. As the result, EESF suppressed iNOS protein expression that may lead to accompany decrease in NO production. Evidently, the anti-inflammatory agents that inhibit NO production and iNOS expression possess the capability to attenuate inflammatory diseases (Lee et al. 2003).

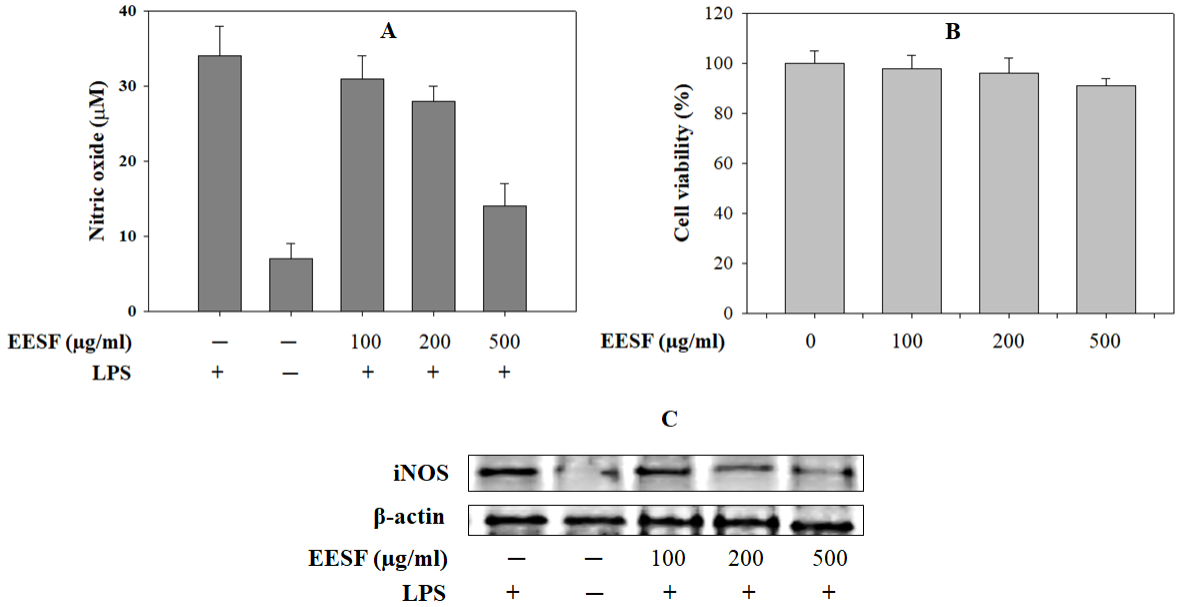

Fig. 2. The anti-inflammatory effect of EESF on LPS-stimulated RAW 264.7 cells. The cells were treated with various concentrations of EESF for 12 h prior to stimulation of LPS for 12 h. (A) Amount of NO was measured using the Griess reaction. Each determination was made in triplicate, and the data are expressed as means ± SD. (C) The protein expression level of iNOS was assessed by Western blotting. β-actin was used as internal controls. (B) The cells were treated with various concentrations of EESF for 24 h. Cell viability was assessed by MTT method, and the results were expressed as percentage of surviving cells over blank cells (no addition of EESF). Each determination was made in triplicate, and the data are expressed as means ± SD. EESF: Ethanol extract Sim fruit.

The Inhibitory Effect of EESF on Histamine Release

Allergy is a disorder of the immune system due to an exaggerated reaction of the immune system to harmless environmental substances, such as animal dander, house dust mites, foods, pollen, insects, and chemical agents. Allergic reaction is characterized by the excessive activation of mast cells and basophils, leading to release various mediators such as histamine and an array of cytokines (Milián and Díaz 2004). Especially, histamine is considered as the major mediator of allergic response, causing pain, acute rhinitis, pruritus, and vasodilatation (Jutel et al. 2009). Thus, histamine is the major target for potential anti-allergic drugs. Herein, anti-allergic activity of EESF was determined via measuring histamine release from calcium ionophore-activated RBL-2H3 mast cells. It was observed that the level of histamine release was significantly reduced by EESF treatment in a dose-dependent manner (**Fig. 3A**). Histamine release level upon treatment with 500 μg/ml of EESF was 44% as compared to control group activated with calcium ionophore alone. Especially, the inhibitory effect of EESF on histamine release was not caused by cytotoxic effect (**Fig. 3B**). It has evidenced that mast cell degranulation and histamine release result in the change of cell membrane and cell morphology (Deng et al. 2009). In this study, the representative images of the mast cells related to cell morphological change were assessed by using light microscopy. In the normal condition, mast cells were observed to be generally branch-shaped with clear membranes, whereas the activated cells induced by calcium ionophore were round-shaped, and had reduced cell size, disrupted boundaries, and irregular surfaces (**Fig. 3C**). Notably, the cell morphology was slightly changed in EESF-pretreated cells before being exposed to calcium ionophore, indicating the inhibitory effect of EESF on mast cell degranulation and histamine release. The reduction of histamine release from the activated mast cells by EESF supported its role as a potential anti-allergic agents. Up to now, numerous potential anti-allergic agents have been found due to inhibition of histamine release (Vo et al. 2012a, 2018a).

CONCLUSION

In conclusion, Sim fruit *R. tomentosa* has been determined as a promising biomaterial with anti-oxidant, anti-inflammatory, and anti-allergic activities. The biological activities have been evidenced via scavenging DPPH and $ABTS^+$ radicals, suppressing NO production and iNOS protein expression from LPS-stimulated RAW 264.7 cells, and inhibiting histamine release from calcium ionophore-activated RBL-2H3 mast cells. However, the further studies related to the evaluation of chemical components, bioactive compounds, and biological activities of Sim fruit *R. tomentosa* are necessary to support its role as a bioactive ingredient for the development of alternative medicine.

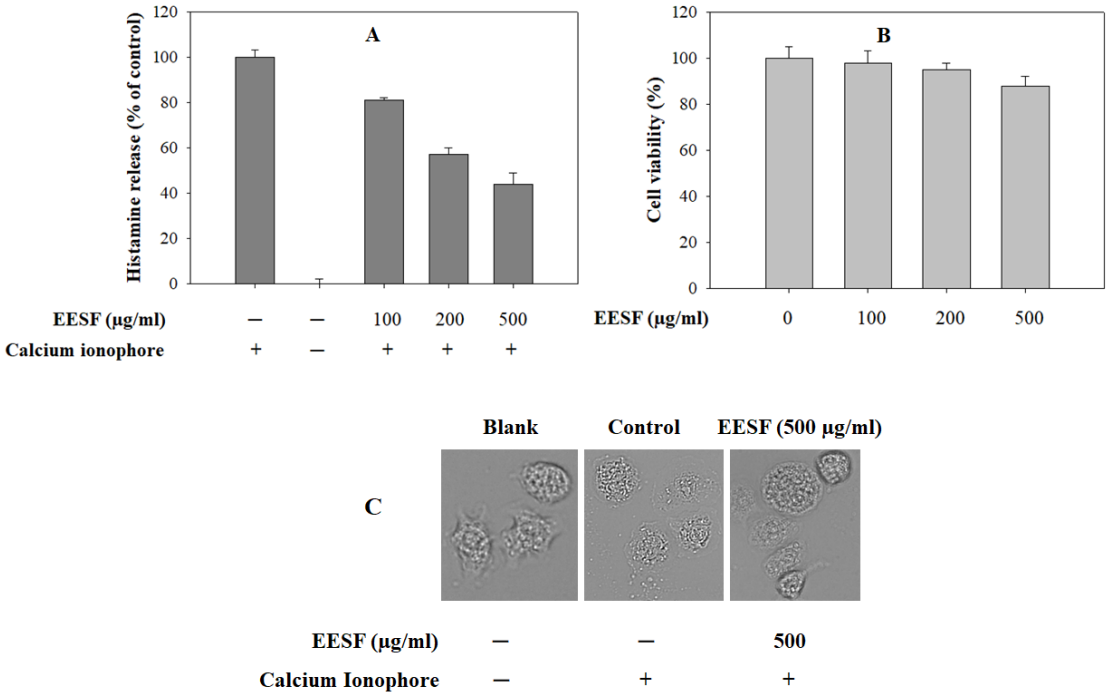

Fig. 3. The anti-allergic effect of EESF on calcium ionophore-activated RBL-2H3 mast cells. The cells were treated with different concentrations of EESF for 12 h before activation of calcium ionophore for 30 min. (A) The level of histamine release was measured via a spectrofluorometric assay. Each determination was made in three independent experiments, and the data are shown as means ± SD. (C) The representative images of the cells were assessed by using light microscopy (magnification, ×20). (B) The cells were treated with various concentrations of EESF for 24 h. Cell viability was assessed by MTT method, and the results were expressed as percentage of surviving cells over blank cells (no addition of EESF). Each determination was made in triplicate, and the data are expressed as means ± SD. EESF: Ethanol extract Sim fruit.

ACKNOWLEDGEMENTS

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106-NN.02-2016.68.

REFERENCES

Cui C, Zhang S, You L, Ren J, Luo W, Chen W, Zhao M (2013) Antioxidant capacity of anthocyanins from Rhodomyrtus tomentosa (Ait.) and identification of the major anthocyanins. Food Chemistry 139: 1-8. https://doi.org/10.1016/j.foodchem.2013.01.107

Dachriyanus, Salni, Sargent MV, Skelton BW, Soediro I, Sutisna M, White A, Yulinah E (2002) Rhodomyrtone, an antibiotic from *Rhodomyrtus tomentosa*. Australian Journal of Chemistry 55: 229-232. https://doi.org/10.1071/CH01194

David B, Wolfender JL, Dias DA (2014) The pharmaceutical industry and natural products: historical status and new trends. Phytochemistry Reviews 14: 299–315. https://doi.org/10.1007/s11101-014-9367-z

Deng Z, Zink T, Chen HY, Walters D, Liu FT, Liu GY (2009) Impact of actin rearrangement and degranulation on the membrane structure of primary mast cells: a combined atomic force and laser scanning confocal microscopy investigation. Biophysical Journal 96: 1629-1639. https://doi.org/10.1016/j.bpj.2008.11.015

Dias DA, Urban S, Roessner U (2012) A Historical Overview of Natural Products in Drug Discovery. Metabolites 2: 303–336. https://doi.org/10.3390/metabo2020303

Gautam R, Jachak SM (2009) Recent developments in anti-inflammatory natural products. Medicinal Research Reviews 29: 767-820. https://doi.org/10.1002/med.20156

Geetha KM, Sridhar C, Murugan V (2010) Antioxidant and healing effect of aqueous alcoholic extract of Rhodomyrtus tomentosa (Ait.) Hassk on chronic gastric ulcers in rats. Journal of Pharmacy Research 3: 2860-2862.

Ghasemzadeh A, Azarifar M, Soroodi O, Jaafar HZE (2012) Flavonoid compounds and their antioxidant activity in extract of some tropical plants. Journal of Medicinal Plants Research 6: 2639-2643. https://doi.org/10.5897/JMPR11.1531

Gordon S (1998) The role of the macrophage in immune regulation. Research in Immunology 149: 685-688. https://doi.org/10.1016/S0923-2494(99)80039-X

Grabowski PS, Wright PK, Van't Hof RJ, Helfrich MH, Ohshima H, Ralston SH (1997) Immunolocalization of inducible nitric oxide synthase in synovium and cartilage in rheumatoid arthritis and osteoarthritis. British journal of rheumatology 36: 651–655. https://doi.org/10.1093/rheumatology/36.6.651

Hazrulrizawati, Hamid and Zeyohannes, Sileshi S, Yusoff MM (2017) *Rhodomyrtus Tomentosa*: A Phytochemical and Pharmacological Review. Asian Journal of Pharmaceutical and Clinical Research 10(1): 10-16.

Jutel M, Akdis M, Akdis CA (2009) Histamine, histamine receptors and their role in immune pathology. Clinical & Experimental Allergy 39: 1786–1800. https://doi.org/10.1111/j.1365-2222.2009.03374.x

Kanwar JR, Kanwar RK, Burrow H, Baratchi S (2009) Recent advances on the roles of NO in cancer and chronic inflammatory disorders. Current Medicinal Chemistry 16: 2373–2394. https://doi.org/10.2174/092986709788682155

Kim JH, Kismali G, Gupta SC (2018) Natural Products for the Prevention and Treatment of Chronic Inflammatory Diseases: Integrating Traditional Medicine into Modern Chronic Diseases Care. Evidence-Based Complementary and Alternative Medicine 2018: 9837863. https://doi.org/10.1155/2018/9837863

Lai TN, André C, Rogez H, Mignolet E, Nguyen TB, Larondelle Y (2015) Nutritional composition and antioxidant properties of the sim fruit (*Rhodomyrtus tomentosa*). Food Chemistry 168: 410-416. https://doi.org/10.1016/j.foodchem.2014.07.081

Le PU, Ngo DH, Vo TS (2018) Optimization of Extraction Conditions for Achieving High Content and Antioxidant Activities of the Total Phenolic Compounds from Phu Quoc Sim Fruit (*Rhodomyrtus tomentosa* (Aiton) Hasak.). Journal of Medicinal Materials 23: 157-166.

Lee H, Kim YO, Kim H, Kim SY, Noh HS, Kang SS, Cho GJ, Choi WS, Suk K (2003) Flavonoid wogonin from medicinal herb is neuroprotective by inhibiting inflammatory activation of microglia. The FASEB Journal 17: 1943–1944. https://doi.org/10.1096/fj.03-0057fje

Lim TK (2012) Edible medicinal and non medicinal plants, 1st ed. Springer, New York.

McAdam E, Haboubi HN, Forrester G, Eltahir Z, Spencer-Harty S, Davies C, Griffiths AP, Baxter JN, Jenkins GJ (2012) Inducible nitric oxide synthase (iNOS) and nitric oxide (NO) are important mediators of reflux-induced cell signalling in esophageal cells. Carcinogenesis 33: 2035-2043. https://doi.org/10.1093/carcin/bgs241

McInnes IB, Leung BP, Field M, Wei XQ, Huang FP, Sturrock RD, Kinninmonth A, Weidner J, Mumford R, Liew FY (1996) Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. The Journal of Experimental Medicine 184: 1519–1524. https://doi.org/10.1084/jem.184.4.1519

Milián E, Díaz AM (2004) Allergy to house dust mites and asthma. Puerto Rico health sciences journal 23: 47-57.

Saising J, Hiranrat A, Mahabusarakam W, Ongsakul M, Voravuthikunchai SP (2008) Rhodomyrtone from Rhodomyrtus tomentosa (Aiton) Hassk. as a natural antibiotic for staphylococcal cutaneous infections. Journal of Health Science 54: 589–595. https://doi.org/10.1248/jhs.54.589

Vo TS, Kim SK (2013) Fucoidans as a Natural Bioactive Ingredient for Functional Foods. Journal of Functional Foods 5: 16-27. https://doi.org/10.1016/j.jff.2012.08.007

Vo TS, Kim YS, Ngo DH, Le PU, Kim SY, Kim SK (2018a) Spirulina maxima peptides suppress mast cell degranulation via inactivating Akt and MAPKs phosphorylation in RBL-2H3 cells. International Journal of Biological Macromolecules 118(B): 2224-2229.

Vo TS, Kong CS, Kim SK (2011) Inhibitory effects of chitooligosaccharides on degranulation and cytokine generation in rat basophilic leukemia RBL-2H3 cells. Carbohydrate Polymer 84: 649–655. https://doi.org/10.1016/j.carbpol.2010.12.046

Vo TS, Le PU, Ngo DH (2018b) The increased gamma-aminobutyric acid content by optimizing fermentation conditions of bacteria from kimchi and investigation of its biological activities. EurAsian Journal of BioSciences 12: 369-376.

Vo TS, Ngo DH, Kang KH, Park SJ, Kim SK (2014) The role of peptides derived from Spirulina maxima in downregulation of FcεRI-mediated allergic responses. Molecular Nutrition & Food Research 58: 2226-2234. https://doi.org/10.1002/mnfr.201400329

Vo TS, Ngo DH, Kim SK (2012a) Marine algae as a potential pharmaceutical source for anti-allergic therapeutics. Process Biochemistry 47: 386-394. https://doi.org/10.1016/j.procbio.2011.12.014

Vo TS, Ngo DH, Ta QV, Wijesekara I, Kong CS, Kim SK (2012b) Protective effect of chitin oligosaccharides against lipopolysaccharide-induced inflammatory response in BV-2 microglia. Cell Immunology 277: 14-21. https://doi.org/10.1016/j.cellimm.2012.06.005

Wu YY, Li W, Xu Y, Jin EH, Tu YY (2011) Evaluation of the antioxidant effects of four main theaflavin derivatives through chemiluminescence and DNA damage analyses. Journal of Zhejiang University SCIENCE B 12: 744–751. https://doi.org/10.1631/jzus.B1100041