Anti-tumor Activity of Nuvastatic™ (C5OSEW5050ESA) of *Orthosiphon stamineus* and Rosmarinic Acid in an Athymic Nude Mice Model of Breast Cancer

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Abstract

The current treatment strategies for metastatic breast cancer depend on the cancer subtype by palliating symptoms and prolonging life. However, triple-negative breast cancers have no targeted treatment available. *Orthosiphon stamineus* (O.s) is a traditional folk medicine plant used in South East Asia to treat many diseases. The aim of this study is to evaluate the anti-tumor activity of O.s extract formulation (ID: C5OSEW5050ESA trademarked as Nuvastatic™) and its major component, rosmarinic acid in a breast in vivo tumor xenograft model. Human triple-negative breast cancer cells were transplanted into the mammary fat pad of 20 athymic nude mice. Fourteen days post-injection, mice were randomly assigned to four groups. C5OSEW5050ESA (200 and 400 mg/kg/day) and rosmarinic acid (32 mg/kg/day) were administered orally. The body weight and tumor size were measured twice a week. Histopathological analyses of tumor tissues were conducted. Tumor necrosis and tumor growth were determined by hematoxylin and eosin staining. A significant reduction in tumor size and growth was found in all treatment groups. No significant difference between C5OSEW5050ESA at 200 mg/kg and rosmarinic acid in the reduction of tumor size and necrosis. However, a more significant reduction was found in tumor growth and necrosis with 400 mg/kg of C5OSEW5050ESA treatment as compared to other treatments. These results highlighted the anti-tumor activity of C5OSEW5050ESA in reducing breast tumor growth in mice compared to the lower dose of C5OSEW5050ESA and rosmarinic acid. This study provides valuable insights into using C5OSEW5050ESA as a treatment to target triple-negative breast cancers in vivo.

Key Words: Breast cancer, *Orthosiphon stamineus*, C5OSEW5050ESA, Rosmarinic acid, Nuvastatic, Medicinal plant, Anti-tumor

Introduction

Metastatic breast cancer remains incurable in almost all patients. The most histological types of breast cancer are invasive ductal carcinoma (IDC) (50% - 75%), invasive lobular carcinoma (ILC) (5% - 15%) and mixed IDC/ILC carcinomas, which represent the remaining patients (Waks & Winer, 2019). The primary diagnosis depends on the histopathology report and the presence or absence
of hormone receptors for progesterone, estrogen, and the human epidermal growth factor receptor-2 (HER2). A high proportion of breast cancers are receptor-positive (77%) and the targeted treatment has proven efficacy. However, in the case of triple-negative breast cancers (Negative for all three receptors), no targeted treatment is available (Dass et al., 2021). Similar basic categories of systemic therapy are used in breast cancers as in neoadjuvant/adjuvant approaches outlined here. Many side effects have been reported with adjuvant chemotherapy for breast cancer patients, such as nausea, loss of appetite, vomiting, hair loss, fatigue, neutropenia, and premature menopause. Consequently, extensive research efforts have been employed to identify the cause of breast cancer onset by identifying the critical molecular mechanism of its progression and treatment with lower and limited toxicity. In the last decade, great efforts are certainly promising since overall survival has greatly improved in several breast cancer types.

Medicinal plant products have gained popularity and are reported to prevent and/or palliate the side effects, improve quality of life, and reduce stress (A. H. Yehya et al., 2017). Orthosiphon stamineus (O.s), Lamiaceae family, is a common Asian medicinal plant used for the treatment of many diseases, including inflammation, urinary tract infections, bacterial infections, jaundice, rheumatism, influenza, and cancer (Tabana et al., 2016; A. H. Yehya, Asif, Majid, & Oon, 2020). A decoction made from leaves of O.s known as “java tea” is commonly consumed for health care and fitness. The safety profile of 50% ethanol extract of O.s has been well established, with LD₅₀ being more than 5000 mg/kg (Mohamed et al., 2011; A. H. Yehya et al., 2019). Several compounds have been isolated from O.s, containing at least 20 phenolic and flavonoid compounds such as rosmarinic acid, eupatorin, and sinesitin; and also pentacyclic triterpenes including betulinic acid, ursolic acid, oleanolic acid, and b-sitosterol. Rosmarinic acid has been classified as one of the most active components in 50% ethanol extract of O.s leaves and is mainly responsible for potential anti-tumor activity of O.s extract (F. Al-Suede et al., 2014; A. H. Yehya et al., 2020). C5OSEW5050ESA (Nuvastatic™) is a proprietary extract of O.s that has completed phase 2/3 clinical studies among cancer patients with solid tumors for cancer fatigue. In a recent study by Yehya et al. (A. H. Yehya et al., 2020) showed that the combination treatment of C5OSEW5050ESA and gemcitabine (Chemodrug) has reduced pancreatic cells proliferation, migration, and colony formation and enhanced apoptosis. Another study has shown that C5OSEW5050ESA has reduced colon tumor size to 70% compared to the vehicle control in an orthotopic colon tumor model in athymic nude mice (F. Al-Suede et al., 2014). However, so far no study has reported anti-tumor effects of C5OSEW5050ESA on breast cancer compared to its bioactive compound, rosmarinic acid. Thus, the current study was intended to evaluate the anti-tumor effects of Nuvastatic™ (C5OSEW5050ESA) and rosmarinic acid using triple-negative breast cancer cells nude mice model.

Materials and Methods

Plant materials
Nuvastatic™ (C5OSEW5050ESA) (Catalogue No. 931886-P) was obtained from NatureCeuticals Sdn. Bhd, Kedah, Malaysia. The extract was prepared according to NatureCeuticals proprietary in house method. The C5OSEW5050ESA was stored carefully in an airtight container for further experiments.

Cell line and chemicals
The transfected human triple-negative breast cancer cells (MDA-MB321-RFP) were purchased from American Type Culture Collection (ATCC, USA). The cells were cultivated in DMEM medium (Nacalai Tesque, USA) along with 10% FBS (Biowest, USA) and 100 units/mL penicillin-streptomycin solution (Nacalai Tesque, USA). Rosmarinic acid (Catalogue No. 536954) was procured from Sigma-Aldrich, Missouri, USA. Matrigel Growth Factor Reduced was purchased from CORNING, USA.

Standardization of C5OSEW5050ESA by UV-HPLC
HPLC analysis was performed using an Agilent 1260 infinity system (Waldronn, Germany) system equipped with a quaternary pump (G1311C), autosampler (G1329B), column oven (G1316A), and ultraviolet detector (G1314F). Standard compounds stock solution was prepared by dissolving 5 mg of each standard in 5 mL of HPLC grade methanol and then filtered through a 0.45 μm filter (Whatman, USA). Serial dilutions of working standard reagents were prepared by diluting the stock solution with HPLC methanol. C5OSEW5050ESA (50 mg) was dissolved in 10 mL of 50% ethanol HPLC grade. The extract solution (0.4 mL) was mixed with 3.6 mL methanol (HPLC grade). All prepared samples were filtered using a 0.45 μm filter (Whatman, USA) and transferred to HPLC vials before injection.

Cell viability assay
Cell proliferation assay was performed in a 96-well plate format using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide) to determine the 50% inhibitory concentration (IC₅₀) of C5OSEW5050ESA and rosmarinic acid. Different doses of C5OSEW5050ESA (25, 50, 100, 200, 400, and 800 μg/mL) and rosmarinic acid (10, 20, 40, 80, 160, and 320 μg/mL) were tested on MDA-MB321-RFP cells for 24 hours.

Animals

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About twenty athymic NCR nu/nu nude mice (23 ± 2 g) aged 4-6 weeks were obtained from EMAN Biodiscoveries Sdn Bhd. The mice were kept under specific pathogen-free (SPF) cages and provided with highly efficient particulate air filters using animal transport units (Allentown, USA). Sterile food, water and bedding were provided, and mice were kept under recommended conditions (12/12 h light/dark cycle, temperature 24 ± 2°C, and 60% humidity). Sterile bedding was changed twice a week. All in-vivo studies were conducted according to ethical guidelines and were approved by the ethical committee (Reference #: AEA-2021-2220-EA (ethic.2220)).

Establishment of the orthotopic tumors
A 90% confluent MDA-MB321-RFP cells cultures in T75 flasks were trypsinized and resuspended in 10 mL fresh medium. The cells were collected after centrifugation for 5 minutes at 1500 rpm. The cell pellet was resuspended in 0.2 mL of complete DMEM culture medium and matrigel (1:2) supplement with 10% FBS and 100 units/mL penicillin-streptomycin (Pen-Strep) solution and kept on ice. The nude mice were implanted orthotopically into the right lower mammary pad 6 × 10^6 cells in 0.2 mL culture medium before returning the mice back to their original cages.

Treatment and tumor size measurement
After two weeks of tumor implantation, four groups of animals (n = 5) were divided randomly. Group 1 served as negative control and received 0.2 mL of distilled water, while groups 2 and 3 were treated orally with 200 and 400 mg/kg body weight of C5EOSEW5050ESA, respectively. Group 4 was treated with rosmarinic acid (32 mg/kg/day). Body weights of animals were recorded prior to the experiment and every three days. The mice were given daily treatment via oral gavage for up to 3 weeks. Tumor dimensions were measured by a digital caliper in 3 angles; length, width and depth (Sayles et al., 2019). The tumor size was calculated by applying the formula: Tumor volume (mm^3) = (length x width x depth) / 2.

Euthanasia and tumor collection
The mice were euthanized using CO2 and followed cervical dislocation at the end of the experiment. The xenograft tumors were harvested and preserved in 4% paraformaldehyde for histopathology evaluation and examined vascularisation, tissue morphology and necrosis/ apoptosis. The slides were dewaxed in xylene and rehydrated through graded alcohol. After rinsing, eosin was added to the slides for 1 min. Slides were rinsed in water and air-dried at room temperature. The slides were then mounted with glycerol (Sigma, USA). Stained slides were examined for the percentage of necrosis within the tumor areas under a light microscope.

Statistical methods
Prism (GraphPad, USA) and Graphing Excel software (Microsoft, USA) were used for statistical analysis. Data were expressed as mean ± S.D for three data sets. The data analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey’s honest significant difference (HSD) post hoc test to assess the significant differences among all groups. A P < 0.05 was considered significant compared to the respective untreated group.

Results
HPLC analysis of C5EOSEW5050ESA
Four marker flavonoids including rosmarinic acid, eupatorin, sinesitin, and 30-hydroxy-5, 6, 7, 40-tetramethoxyflavone were quantitatively determined using HPLC method. The HPLC chromatograms and the contents of the mixed standard of bioactive markers in C5EOSEW5050ESA (absorbance at 330 nm) are shown in Figure 1. The regression equations of these curves and their coefficients of determination (R^2) were calculated as follows: Rosmarinic acid: y = 34556x + 3794.8, R^2 = 0.9999; Eupatorin: y = 41081x + 6754.2, R^2 = 0.9999; Sinesitin: y = 70658x + 22281.0, R^2 = 0.9999; and 30-hydroxy-5,6,7,40-tetramethoxyflavone: y = 43060x + 12967.0, R^2 = 0.9999. Furthermore, the standards curves were used to calculate the contents (%) of the four marker compounds in C5EOSEW5050ESA as represented in Table 1. Thus, to calculate the % of the markers (wt/wt) = (the found concentration / concentration of injected sample) x 100.

C5EOSEW5050ESA and rosmarinic acid reduced MDA-MB321-RFP cell viability
The IC₅₀ values of C5EOSEW5050ESA and rosmarinic acid towards MDA-MB321-RFP cells were estimated to be 370 µg/mL and 300 µg/mL respectively (Figure 2).

Anti-tumor activity of C5OSEO5W5050ESA and rosmarinic acid on tumor size and weight
The results showed that mice treated with C5OSEO5W5050ESA showed a dose-dependent reduction in tumor size. Figure 3 shows the tumor size in the respective treated animals. The C5OSEO5W5050ESA at 200 and 400 mg/kg exhibited a dose-dependent suppression of MDA-MB 321-RFP tumor size with 73.1 ± 3% and 82 ± 2.2%, respectively, compared to the vehicle control animals. Rosmarinic acid reduced the tumor size to 76.2 ± 4% compared to control group.
Table 1. Contents of four bioactive compounds in C5EOSEW5050ESA.

<table>
<thead>
<tr>
<th>Component</th>
<th>C5EOSEW5050ESA (%)</th>
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<tbody>
<tr>
<td>Rosmarinic acid</td>
<td>6.90</td>
</tr>
<tr>
<td>Eupatorin</td>
<td>0.77</td>
</tr>
<tr>
<td>Sinesitin</td>
<td>0.23</td>
</tr>
<tr>
<td>30-hydroxy-5,6,7,40-tetramethoxyflavone</td>
<td>0.03</td>
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Figure 1. HPLC chromatograms. A) HPLC chromatogram of standard markers, rosmarinic acid (RA), eupatorin (EUP), sinesitin (SIN), and 30-hydroxy-5,6,7,40-tetramethoxyflavone (TMF). B) HPLC chromatograms of C5OSEW5050ESA showing different proportions of respective markers.

Figure 2. Cell viability assay of C5OSEW5050ESA and rosmarinic acid on MDA-MB321-RFP cells.

Figure 3. The anti-tumor effect of C5OSEW5050ESA and rosmarinic acid on triple-negative breast tumor size and weight. C5OSEW5050ESA at 200 and 400 mg/kg and rosmarinic acid reduced the size of the tumors significantly as compared to the negative control group. Statistics analysis ($P^* < 0.05; P^{**} < 0.01; P^{***} < 0.001$, One-way ANOVA, n = 5 animals per group) using GraphPad Prism 6.0 software.
C5EOSEW5050ESA and rosmarinic acid synergistically inhibited triple-negative breast tumor growth in the xenograft model

C5EOSEW5050ESA at 200 mg/kg or 400 mg/kg and rosmarinic acid (32 mg/kg) inhibited tumor growth compared to the vehicle untreated group (Figure 4). There is a significant difference in reducing tumor growth between the low and the high dose of C5EOSEW5050ESA. Furthermore, there were no significant differences in inhibition of tumor growth between C5EOSEW5050ESA at 400 mg/kg and rosmarinic acid (Figure 4).

C5EOSEW5050ESA and rosmarinic acid synergistically enhanced triple-negative breast tumor necrosis in the xenograft model

C5EOSEW5050ESA at 200 mg/kg or 400 mg/kg and rosmarinic acid (32 mg/kg) enhanced tumor necrosis compared to the vehicle untreated group (Figure 5). The percentage of tumor necrosis in C5EOSEW5050ESA at 200 mg/kg, C5EOSEW5050ESA at 400 mg/kg, and rosmarinic acid 32 mg/kg were 35%, 65%, and 45% respectively compared to the vehicle untreated animals.

C5EOSEW5050ESA and rosmarinic acid demonstrated no effect on the body weight of mice

There were no significant differences in the average of body weight among all the groups treated by C5OSEW5050ESA (200 or 400 mg/kg) and rosmarinic acid (32 mg/kg) compared to the vehicle control (Figure 6).

Discussion

The current study describes the anticancer effects of C5OSEW5050ESA (Nuvastatic™) ethanol extract of O. s and rosmarinic acid, a marker compound of C5OSEW5050ESA in non-hormone dependent triple-negative breast cancer. HPLC results revealed that rosmarinic acid is the major component of C5OSEW5050ESA as compared to other bioactive compounds (Figure 1). The results revealed that animals treated with low and high doses of C5OSEW5050ESA exhibited dose-dependent reduction in triple-negative breast tumor size (Figure 3) whereas treatment at low dose and high dose showed a dose-dependent suppression of triple-negative breast tumor growth with 73.1 ± 3% and 82 ± 2.2%, respectively, as compared to the vehicle control group. Meanwhile, rosmarinic acid reduced the tumor size to 76.2 ± 4% compared to the control group. The concentration of rosmarinic acid used in this study is equivalent to that present in the high dose of the extract (7%) per day, which can elaborate the effect of rosmarinic alone compared to C5OSEW5050ESA. These results showed that rosmarinic acid plays a vital role in the effect of C5OSEW5050ESA compared to eupatorin (0.77%) and sinesetin (0.23%) due to the high content of rosmarinic acid in C5OSEW5050ESA. The C5OSEW5050ESA contains rosmarinic acid and other bioactive compounds in different concentrations that may give a synergetic effect to C5OSEW5050ESA.

This study revealed that both C5OSEW5050ESA and rosmarinic acid inhibited tumor growth compared to the vehicle control group (Figure 4). C5OSEW5050ESA has been shown to significantly inhibit the tumor growth even at a low dose. No significant differences have been found between rosmarinic acid and a high dose of C5OSEW5050ESA in the inhibition of tumor growth and weight. Previously, rosmarinic acid has been displayed anticancer, apoptotic, and anti-lipid peroxidative effects in 7,12-dimethylenz(a)anthracene-induced skin carcinogenesis of Swiss albino mice (Sharmila & Manoharan, 2012). These findings could possibly explain the diminished cells viability in triple-negative breast tumor cells in the current study either by rosmarinic acid and C5OSEW5050ESA (Figure 2 and 4). Also, C5OSEW5050ESA has the ability to reduce triple-negative breast tumor growth and size. These data are consistent with the finding found with the C5OSEW5050ESA ethanol extract, which revealed no tumor cell growth compared to the control group using xenograph method of tumor models transplanted with breast cancer cells (MCF7) and colon cancer cells (HCT116) (Ahamed, Ismail, Aisheh, Abdul Majid, & Majid, 2010). In addition, C5OSEW5050ESA exerted anti-angiogenic activity by inhibiting the Vascular Endothelial Growth factor (VEGF), Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Interleukin 2 (IL-2) & Interleukin 7 (IL-7), Nerve Growth Factor β (NGF-β), Transforming Growth Factor -α (TGF-α) and Tumor Necrosis Factor- β (TNF-β) (Al-Suede et al., 2021). C5OSEW5050ESA also has been demonstrated to cause significant upregulation of interferon α (IFN-α), interferon β (IFN-β), interferon γ (IFN-γ) and Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Al-Suede et al., 2021).

Detection of tumor necrosis in breast cancer can provide predictive data suggesting early recurrence or death, and is used in treatment planning (Gilchrist, Gray, Fowble, Tormey, & Taylor, 1993). As such, C5OSEW5050ESA and rosmarinic acid were tested for their effects on tumor necrosis. Hence, the results revealed that the xenograft model showed that C5OSEW5050ESA and rosmarinic acid synergistically enhanced breast cancer tumor necrosis. It was found that C5OSEW5050ESA at a low dose boosted tumor necrosis by 35%, while rosmarinic acid was improved by 45% (Figure 5).

Interestingly, C5OSEW5050ESA at a high dose boosted tumor necrosis highly by 65% (Figure 5). It has been reported that the high existence of methoxylated flavonoids (eupatorin and sinesetin) in O.s extract reveals the potential cytotoxic effect of C5OSEW5050ESA. Eupatorin was identified as an effective inhibitor for in vitro proliferation of breast cancer cells.
Figure 4. Tumor growth curve of Panc-1 cells in orthotopic triple-negative breast tumor growth in nude mice. Treatments were given when the tumors reached 100 mm³. C5OSEW5050ESA at 200 mg/kg, 400 mg/kg, and rosmarinic acid significantly reduced tumor weight compared to vehicle control. Error bars represent SD. Statistical analysis (P *< 0.05; P ** < 0.01; P *** < 0.001, exponential growth equation, Fit, One-way ANOVA with Tukey’s HSD post hoc test, n = 5 animals per group) using GraphPad Prism 6.0 software.

Figure 5. Tumor necrosis percentage in tumor tissues stained with haematoxylin and eosin (n=5, x10). C5OSEW5050ESA at 200 mg/kg and 400 mg/kg, and rosmarinic acid at 32 mg/kg synergistically enhanced tumor necrosis compared to the vehicle treatment. Arrows indicate the necrotic area in tumor sections.

Figure 6. The effect of C5OSEW5050ESA and rosmarinic acid on the body weight of mice. No significant differences in the average of body weight in all treated groups compared to the vehicle control.
This study gives preliminary scientific evidence about the safety profile of Nuvastatic™ (C5EOSEW5050ESA) and rosmarinic acid in an athymic nude mouse model. Taken together, our results suggest the cumulative anticancer effect of C5EOSEW5050ESA on in vivo non-hormone dependent breast cancer while confirming our previous experiments that suggested mechanisms for the anticancer properties of C5EOSEW5050ESA. Our study expands insights into the anti-triple negative breast cancer properties of C5EOSEW5050ESA and provides an alternative for the prevention and therapeutics of non-hormone dependent breast cancer. Therefore, Nuvastatic™ has the potential to be employed as an anticancer treatment to treat triple-negative breast cancer.

Conclusion

This study gives preliminary scientific evidence about the safety profile of Nuvastatic™ (C5EOSEW5050ESA) and rosmarinic acid in an athymic nude mouse model. Taken together, our results suggest the cumulative anticancer effect of C5EOSEW5050ESA on in vivo non-hormone dependent breast cancer while confirming our previous experiments that suggested mechanisms for the anticancer properties of C5EOSEW5050ESA. Our study expands insights into the anti-triple negative breast cancer properties of C5EOSEW5050ESA and provides an alternative for the prevention and therapeutics of non-hormone dependent breast cancer. Therefore, Nuvastatic™ has the potential to be employed as an anticancer treatment to treat triple-negative breast cancer.

Author Contributions

AHSY and AMSA have designed the experiments. AHSY, FSRA, SBS, MAA, NAA have conducted the experiments. AHSY, MAA, and SFJ have drafted the manuscript. AHSY, MAA, and MJAA have analyzed the data.

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Competing financial interests

All the authors of the manuscript, except Amin Malik Shah Abdul Majid, declare no financial and non-financial competing interests. Amin Malik Shah Abdul Majid has a commercial interest in a company that owns the intellectual property of Nuvastatic.

Supplementary Information

None

References


Sharmila, R., & Manoharan, S. (2012). Anti-tumor activity of rosmarinic acid in 7, 12-dimethylbenz (a) anthracene (DMBA) induced skin carcinogenesis in Swiss albino mice.


