Antiangiogenic and Anticancer Potential of Supercritical Fluid Extracts from Nutmeg Seeds; *In vitro, Ex vivo* and *In silico* studies

Sawsan S. Al-Rawi[1*], Ahmad Hamdy Ibrahim[2], Marthad A. Hamde[3], Dinesh Babu[4], Mansoureh Nazari[5], Mohd Omar Ab Kadir[6], Aman Shah Abdul Majid[7] and Amin Malik Shah Abdul Majid[8]

Abstract

Background: Angiogenesis is a vital process of forming new blood vessels that occurs during several normal physiological processes. This study aimed to investigate the antiangiogenic and cytotoxic properties of nutmeg extracts derived by Soxhlet and supercritical fluid extraction (SFE) with an *in-vitro*, *ex-vivo*, and *in-silico* studies. **Method:** Nutmeg anticancer property was evaluated against breast cancer cell MCF7 and colon cancer cell HCT116 with MTT in-vitro assay. The antiangiogenic property was investigated using 3D ex-vivo rat aorta assays. The chemical composition of nutmeg extract was characterized using GC/TOF-MS. Subsequently, the main compounds in the nutmeg extract were analyzed against the angiogenesis-associated molecules (COX-1, VEGFA, HIF, and EGF) by molecular docking and were compared with tamoxifen and 5-fluorouracil. **Results:** The SFE extracts exhibited higher antiangiogenic properties than the Soxhlet (IC50 31µg/mL). Nutmeg SFE extract exhibited higher cytotoxicity towards HCT116 than MCF7 cells. Several active compounds, including myristicin, eugenol, safrole, and α-asarone were identified in the nutmeg SFE extracts using GC/TOF-MS. Molecular docking revealed strong interactions between these compounds and angiogenesis molecular mediators. Particularly, myristicin blocked COX-1, VEGFA, HIF, and EGF enzymes, indicating possible binding interactions. **Conclusion:** Myristicin, α-asarone, safrole, and eugenol work synergistically to induce antiangiogenic effects, making nutmeg a promising natural source of angiogenesis inhibitors for future anticancer therapies. The Molecular docking result confirmed that the inhibition of COX-1, VEGF-A, HIF, and EGF by nutmeg compounds offers great potential in the treatment and prevention of various angiogenesis diseases.

Keywords: Angiogenesis; COX-1; VEGF; HIF; EGF; Cytotoxicity; Cancer; MCF7; HCT116; GC-TOF/ MS, and Nutmeg.

Significance | Understanding of nutmeg's biological activity and its possible applications in cancer therapy.

*Correspondence: Dr. Sawsan S. Al-Rawi; Department of Biology Education, Faculty of Education, Tishk International University, Erbil, KRG/ Iraq. Sawsan.hamed@tiu.edu.iq; Tel.: +9647502268571.

Editor Asaduzzaman Shishir And accepted by the Editorial Board Nov 14, 2023 (received for review Oct 25, 2023)

Introduction

Angiogenesis is a crucial process of forming new blood vessels involved in the initial development of embryonic growth, wound healing, tissue development, and many diseases, including cancer Rust, 2020; Ibrahim et al., 2013). Angiogenesis plays an essential

Author Affiliation:
[1]*Department of Biology Education, Faculty of Education, Tishk International University, Erbil, KRG/ Iraq. Sawsan.hamed@tiu.edu.iq
[2] Department of Pharmacy, Faculty of Pharmacy, Tishk International University, Erbil, KRG/ Iraq. ahmad.hamdy@tiu.edu.iq
[3] School of Computer Sciences, Universiti Sains Malaisia (USM)
[4] Faculty of Pharmacy & Pharmaceutical Sciences, Katz Group-Rexall Centre for Pharmacy and Health Research, University of Alberta, Edmonton, AB, T6G 2E1, Canada.
[5] Pharmacy School, 17August 1945 University, Jakarta, Indonesia.
[6] Environmental Technology department, School of industrial technology, Universiti sains Malaysia
[7] Faculty of Medicine, Department of Pharmacology/Centre for Angiogenesis and Natural Product Research
[8] Eman biodiscoveries, Sdn Bhd and ACRF Department of Cancer Biology and Therapeutics, The John Curtin School of Medical Research, Australian National University, Acton, Australia

Please cite this article:
Sawsan S. Al-Rawi, Ahmad Hamdy Ibrahim, Marthad A. Hamde et al., (2023), Antiangiogenic and Anticancer Potential of Supercritical Fluid Extracts from Nutmeg Seeds; *In vitro*, *Ex vivo* and *In silico* studies, Journal of Angiotherapy, 7(1), 1-13, 9371

in tumor growth by supplying oxygen and nutrients for the proliferating tumor (Al-Ostoot et al., 20221. Certain types of normal cells and several cancer cells produce some factors, such as vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor, which play important roles in tumor angiogenesis in addition to hypoxia (Hamdi et al., 2023; Montemagno & Pagès, 2020). The abnormal angiogenesis has been strongly linked to the disease pathogenesis (Al-Rawi, et al., 2023). Normally, in healthy tissues, the proangiogenic and antiangiogenic factors are balanced; however, in tumors, angiogenesis is stimulated when tumor tissues require nutrients and oxygen. The proangiogenic factors are overexpressed in several cells' types, including cancer cells, which causes blood vessels to be structurally and functionally aberrant leading to hypoxia. However, the suppression of proangiogenic factor signaling pathways can shift or reverse the balance of angiogenic factors and normalize the vasculature, which helps to normalize any condition (Yang, et al., 201). Thus, angiogenesis inhibition offers a potential therapeutic strategy in treating many diseases, including cancer, by targeting vessel growth or blocking the action of angiogenesis inducers. Cancer has become the second biggest fatal disease around the world and the biggest cause of death worldwide (Mattiuzzi C, Lippi G. (2019). Moreover, cancer treatment is quite expensive and current cancer treatments are not pretty efficient as they cause unpleasant responses and many adverse reactions to various body parts (Baldo, et al., 2015). Therefore, due to the efficiency and potency of angiogenesis inhibitors, the Food and Drug Administration (FDA) has authorized a number of them for the treatment of cancer (Lugano, et al., 2020). In 2004, Bevacizumab (Avastin) was the first antiangiogenic medication to be licensed by the FDA for the treatment of metastatic colorectal cancer, which is a recombinant humanized monoclonal antibody of VEGFA, an isoform of VEGF. Since then, studies involving the search for angiogenesis inhibitors are always on the rise. As nature offers us vast resources of high therapeutic potential, finding new antiangiogenic compound[s] extracted from natural sources could provide us with promising novel therapies to treat cancer. Many active compounds extracted from plants have been shown to have antibacterial and anticancer effects with other biological properties (Hanif, et al., 2023; Wang, et al., 2012). Myristica fragrans is originally from the Indonesian Banda Islands. It is a tropical evergreen tree that is grown in most of the Southeast Asian nations, including Malaysia, India, Indonesia, Grenada, Vietnam, Sri Lanka, and Grenada. The dried kernel of the Myristica fragrans seed, known as nutmeg, has a warm, sweet flavor and pleasant scent and is used as a spice throughout the world (Khan, et al., 2019; Ha, et al., 2020). Most ancient civilizations have employed it for centuries in their traditional medicine. Nutmeg is a source of a variety of bioactive molecules implicated in the treatment of several diseases (Barman, et al., 2021). Among the broad spectrum of biological properties attributed to the use of nutmeg, its anticancer properties have been of recent increasing interest. Moreover, our preliminary investigations on the extracted oil following the supercritical fluid extraction of nutmeg showed a potential antiangiogenic property (Al-Rawi, et al., 2911; Ibrahim & Al-Rawi, 2018). So, this work aimed to further investigate the cytotoxic and antiangiogenic activities of nutmeg seed extracts as well as to predict the molecular interaction mechanism of the main active compounds of nutmeg seed extract with the molecular mediators involved in angiogenesis.

2. Methods

2.1 Nutmeg seed extraction

The preparation and extraction of the nutmeg seed samples were carried out using supercritical fluid extraction (SFE) according to the method described by (Ab Rahman et al. 2012). The SFE method was chosen to extract the nutmeg seed due to its effective characteristics in extracting the bioactive compounds (Al-Rawi, et al., 2013). Thus, the operation parameters were selected based on the previously reported study (Ibrahim, & Al-Rawi, 2018).

2.2 Identification of the nutmeg constituents using GC/TOF-MS

The primary chemical components that are present in the supercritical extract of nutmeg seeds were identified using gas chromatography/time-of-flight-mass spectrometry (GC/TOF-MS) and compared to the Soxhlet extraction. The gas chromatogram 7890 (Agilent Technologies) and 7890 Series autosampler injector with controller instrument was utilized in this work. The system was coupled with a LECO Pegasus III GC/TOF-MS along with an electron impact ionization apparatus. LECO ChromaTOF® software was used to analyze and evaluate all the data. The analytical procedure was followed in accordance with the procedure previously described (Ibrahim, & Al-Rawi, 2018).

2.3 Cell lines and growth conditions

ScienCell Research Laboratories in the USA provided the human colorectal carcinoma HCT116 and breast cancer MCF7 cell lines for this study. Cells were cultivated at 37o C with 5% CO2 in 25 cm2 flasks (Nunc Brand, Denmark) that contained 5 mL of RPMI-1640 (Gibco, USA) with the addition of 5% and 1% of fetal bovine serum and penicillin-streptomycin respectively. In addition, 0.5 mL of trypsin (Nutricell, Campinas, Brazil) was added to the culture flasks to detach the adherent cell lines. Trypsin was then rendered inactive by adding 5 mL of RPMI 1640 containing 5% fetal bovine serum. Cells were gently pipetted together to facilitate the separation of the cells in the solution. Before plating the cells onto cell culture plates, they were counted and diluted to the proper seeding densities. The compounds were initially screened at one concentration of 100 ug/mL in triplicate wells. Dimethyl

sulfoxide (DMSO) from (Sigma) was used to dissolve all the SFE-extracted samples before RPMI was added at a final concentration of 100 ug/mL. DMSO-containing media was used to treat the cells as a negative control.

2.4 Cytotoxicity Assay

The cytotoxicity of the nutmeg seed extracts was carried out by MTT assay using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (according to the method described by (Dahham, et al., 2018). In brief, the HCT116 and MCF7 cells were seeded in a 96-well microplate (Nunc MicroWell, Thermo Scientific, USA) and were incubated for 24 h at 37°C in 5% CO2. Cells at 70–80% confluence were treated with the SFE extracted samples using a concentration range of 25-200 µg/mL. Subsequently, after 48 h incubation, the cells were treated with the MTT solution and incubated for 4 h at 37°C in 5% CO2. Afterward, the absorbance was measured at a primary wavelength of 570 nm and a reference wavelength of 620 nm; the absorbance was measured using a plate reader called the Infinite® 200 PRO (Tecan Life Sciences, Switzerland). The inhibitory effect of 50% of cell growth (IC50) was calculated to determine the profile of growth inhibition by each extract. Five concentrations of the extracted samples were prepared by serial dilution, which were examined against the growth of two types of cancer cell lines, colon cancer cell HCT116 and breast cancer cell MCF7. The samples concentration ranged from 12.5, 25, 50, 100 and 200 µg/mL.

2.5 Antiangiogenic Assay (3D Ex Vivo Rat Aorta)

The antiangiogenic activity of the nutmeg seed extracts was assessed by the 3D rat aorta assay according to the procedure previously described (Al-Rawi, et al., 2011). In brief, in a 48-well plate, the aorta rings were seeded and treated using a range of concentrations of nutmeg seed SFE extracts and incubated for five days. Subsequently, after a 5-day incubation period, the outgrowths of the new micro-vessel from the aortic rings were measured and quantified compared to the control. An inverted light microscope equipped with a camera with a Leica Quin computerized imaging system was used to quantify the explant growth at a 4x magnification power. The result was calculated using Leica QWin software packages, where the data were presented as mean ± standard deviation. Subsequently, the dose-dependent angiogenic effect of nutmeg SF extracts were investigated using six serial concentrations. Six dilutions were prepared from the selected SFE of nutmeg extract at the following concentrations 25, 50, 75, 100, 175 and 200 µg/mL. All animal experiments were carried out in accordance with the regulations and approval granted by the Animal Ethics Committee, with Ref no. 2010/61/252 from the School of Pharmaceutical Sciences, Universiti Sains Malaysia, Malaysia.

2.6 Molecular Docking Analysis

The docking analyses were carried out to understand the possible molecular interaction of some active compounds in the extract with the molecular mediators involved during angiogenesis (Ibrahim, et al., 2016). The active compounds present in the nutmeg seed extract were carefully evaluated and chosen based on their substantial pharmacological properties related to the antiangiogenic and anticancer activities. Therefore, only the most active compounds in the nutmeg seed extract were selected, evaluated, and compared based on molecular docking attributes with 5-fluorouracil (5-FU) and tamoxifen as positive compounds.

2.7 Docking Software

The Python language software was used as a tool to analyze the docking data obtained from www.python.com. Other tools like the Molecular graphics laboratory (MGL) http://mgltools.scripps.edu, AutoDock4.2 (http://autodock.scripps.edu). Discovery Studio Visualizer 2017, BIOVIA Draw, and Chem3D were also used and downloaded from http://accelrys.com, http://accelrys.com, and https://acms.ucsd.edu, respectively.

2.8 Protein preparation

The structure (3D crystal) of anticancer targets VEGFA (PDB ID: 4KZN), epidermal growth factor (EGF) with PDB ID: 1JL9, cyclooxygenase-1 (COX-1) with PDB ID: 1EQH, and hypoxia-inducible factor alpha (HIF-1α) with PDB ID: 1YCI from Protein Data Bank (www.rvcsb.org/pdb) (**Fig. 4E**) (Habib, et al., 2019). ArgusLab program was used to remove all non-essential water molecules, heteroatoms and the complexes attached to the receptor molecule. The hydrogen atoms were supplied to the targeted molecule receptor.

2.9 Ligand preparation

The identified crystallography structure of myristicin, the main active compound in nutmeg, with the highest quantity in our extracts, was adopted from PubChem and was converted to PDB using PyMOL to make .sdf format to be used in docking analysis. The AutoDock tools were used to prepare protein structures (Nazari, et al., 2019). The protein starting structure was supplied with Kollman charges and polar hydrogen after removing the water molecule. At the binding location, the grid box and grid spacing were set to 126126126 Å and 0.375 Å, respectively. BIOVIA Draw was used to create the first structure for myristicin as a ligand. Positive controls in this study included 5-FU and tamoxifen. Their structures were obtained from the PubChem website, and Gasteiger charges were given to optimal ligands with the use of AutoDock tools 19. Approximately 100 docking runs with a mutation rate of 0.02 and a crossover rate of 0.8 were conducted. A random selection of population size was set at 250 individuals. The search strategy was a Lamarckian genetic algorithm with a translational step of 0.2 Å, a quaternion step of 5 Å, and a torsion step of 5 Å (Nazari, et al., 2021).

2.10 Statistical Analysis

The experiments (except the docking studies) were repeated three times, the data obtained were subjected to ANOVA, and the *P*-value ≤ 0.5% was only considered as statistically significant. The Minitab 14 software was used for all the statistical analysis.

3. Results

In this study, the nutmeg seeds were extracted using SFE and Soxhlet methods. A total of 12 SFE-extracted samples in addition to another Soxhlet extract of nutmeg seeds were collected and screened for their chemical components using GC/TOF-MS. The result of the major chemical constituents presents in nutmeg seed extracts using GC/TOF-MS is represented in Table 1.

3.1 Growth inhibition profile of HCT116 and MCF7 cells using nutmeg extracts.

The results showed that the extracted samples exhibited cytotoxic properties against HCT116 and MCF7 cancer cell lines. However, the IC50 results confirmed that all the SFE-extracted samples of nutmeg seeds were not specific for HCT116 and MCF7 cells significantly, with the exception of the SFE-extracted sample at a 50°C temperature and extraction presuuse of 20.7 MPa, as shown in Table 2. The nutmeg seeds SFE extract at a temperature of 50°C and extraction pressure of 20.7 MPa exhibited an inhibitory effect with an IC50 of 167 and 175 μg/mL on the HCT116 and MCF7 cells, respectively, as shown in Fig. 1, IA and IB. This extract inhibited HCT116 and MCF7 growth four times higher than the Soxhlet extract and two times higher than the nutmeg seed SFE extract at 40°C, as shown by the IC50 (Table 2). Interestingly, the same sample (extracted at temperature of 50°C and pressure of 20.7 MPa) showed a higher effect on the HCT116 than on MCF7 cells. The IC50 results of both SFE- and Soxhlet-extracted samples are shown in Table 2.

3.2 The interaction effect of Extraction Parameters on the inhibition properties of nutmeg extract

In this study, the SFE extraction parameters, such as temperature and pressure, were found to work independently. However, the interaction between pressure and temperature did not significantly affect the viability of HCT116 cells, with a *P*-value of 0.356 (Table 3a). Fig. 1-II illustrates the effects of the interaction between extraction pressure and temperature on the cell growth inhibition using nutmeg seed SFEs. As represented in Fig. 1-II, the extracted sample of the nutmeg seeds showed the highest inhibitory effect at an operating temperature of 50°C and pressure of 20.7 MPa. Fig. 1-IIb shows the interaction behavior of the pressure and temperature on the growth inhibition of MCF7 cells using nutmeg seed SFE extract. The plot indicates that the growth inhibition of MCF7 cells was significantly affected by the interaction of the extraction temperature and pressure. The interaction between temperature and pressure of the SFE extraction of nutmeg seeds on the growth inhibitory effect of MCF7 cells was displayed in Fig. 1-IIb. The statistical analysis showed that the extract at an operating pressure and temperature of 20.7 MPa and 50°C, respectively, has the highest inhibitory effect on MCF cells (Fig. 1-IIB, III). This effect dropped abruptly with increasing the pressure from 20.7 MPa to 27.6 MPa. Interestingly, increasing the pressure to 34.5 MPa resulted in a similar inhibitory property of the extract. However, the inhibitory effect of the nutmeg SFE extract reduced significantly with further pressure increments up to 41.4 MPa. Moreover, the interaction between temperature of 60° and same pressure showed a less inhibitory effect.

3.3 The influence of SF Extraction Parameters independently on the Inhibitory property of Nutmeg extract

The effects of the extraction temperature and pressure separately on the HCT116 cell growth inhibition are presented in Fig. 3a. It can be seen that increasing the temperature of the SF extraction from 40°C to 50°C increased the inhibitory effect of the nutmeg seed SFE extracts steadily. As a result, the cancer cell growth inhibition increased by about 10%. However, when the extraction temperature was increased to 60°C, the inhibitory effect of the nutmeg seed SF extract did not increase further. Yet, the effect of extraction pressure on the inhibitory effect of the nutmeg extract on HCT116 cells was on the contrary, as with increasing the pressure, the extract inhibitory effect was dropped, while the cell growth inhibition decreased steadily with increasing pressure (Fig. 3a). On the other hand, the effect of temperature and pressure independently on the MCF7 cell growth is presented in Fig. 3b. It can be noted that the two parameters (pressure and temperature) of the extraction process could influence the inhibitory effects of the derived extracts independently.

3.4 Statistical Analysis result

The results of the variance analysis of the effect of pressure and temperature on the MCF7 cell inhibition using the nutmeg seed extract are presented in Table 3. The result of ANOVA showed both pressure and temperature significantly affected the cell growth inhibition (based on cell viability percentage) of HCT116 and MCF7 cells with *P*-value < 0.05, as shown in Table 3a and Table 3b, respectively.

3.5 Antiangiogenic properties of SF and Soxhlet extracts of nutmeg seeds.

The result of the experiment shows that all SFE extracts of nutmeg seeds which were extracted at temperatures of 40, 50 and 60°C under different pressures, showed an effective and potent antiangiogenic property at 200 μg/mL more than Soxhlet. Interestingly, the formation of blood vessels at the surface of the rat aorta rings was inhibited significantly compared to the negative and positive controls using this concentration of SF nutmeg seed extract (Fig. 2).

Table 1. List of the major chemical compounds of nutmeg seed extract derived by supercritical fluid extraction.

No	Compound Name	Formula
1	Eugenol	C7H8O2
2	Isoeugenol	C10H12O2
3	Methoxyeugenol	C11H14O3
4	Methyleugenol	C11H14O2
5	Myristicin	C11H12O3
6	α-Asarone	C12H16O3
7	Safrole	C10H10O2
8	Decanoic acid, methyl ester	C11H22O2
9	α-Phellandrene	C10H16
10	Terpinolene	C10H16
11	Sabinene	C10H16
12	4-Carene	C10H16
13	Anisole	CH3OC6H5.
14	α-Thujene	C10H16
15	α-Pinene	C10H16
16	γ-Terpinene	C10H16
16	Copaene	C10H16

Table 2. The IC50 inhibition of SFE- and Soxhlet-derived nutmeg seed extracts on the HCT116 and MCF7 cell lines at a varied range of extraction temperature.

Sample	Temperature (°C)	HCT116 IC50 (μg/mL)	MCF7 IC50 (μg/mL)
SFE extract	60	190	203
SFE extract	50	167	175
SFE extract	40	224	289
Soxhlet extract	40-60	481	488

Table 3a. Analysis of variance for HCT116 cells using adjusted SS for tests

Source	DF	Seq SS	MS	F	P
Temperature	2	2860.39	1430.19	295.90	0.0001
Pressure	3	382.33	127.44	26.37	0.0001
Temperature & Pressure	6	33.83	5.64	1.17	0.3560
Error	24	116.00	4.83		
Total	35	3392.56			

DF; Degrees of freedom, SS; Sum-of-squares, MS; Mean squares, F; F ratio, P; P value

Table 3b Analysis of Variance for MCF7 cells using Adjusted SS for tests

Source	DF	Seq SS	MS	F	P
Temperature	2	60.667	30.333	11.87	0.0001
Pressure	3	239.333	79.778	31.22	0.0001
Temperature& Pressure	6	48.667	8.111	3.17	0.0190
Error	24	61.330	2.556		
Total	35	410.000			

Table 4. Free binding energy (FBE) and inhibition constant (Ki) of each compound after interaction with individual marker molecule.

Compound	FBE-VEGF (Kcal/mol)	Ki (μM)	FBE-EGF (Kcal/mol)	Ki (μM)	FBE-HIF (Kcal/mol)	Ki (μM)	FBE-COX1 (Kcal/mol)	Ki (μM)
Tamoxifen	-7.22	5.13	-6.58	14.92	-7.57	2.84	-10.18	34.38
5-fluorouracil	-6.71	12.12	-4.97	227.34	-5.26	138.96	-5.09	187.1
Myristicin	-4.57	443.74	-5.7	66.69	-5.82	53.81	-7.2	5.25
α-asarone	-4.55	461.44	-5.71	65.65	-5.07	190.68	-6.75	11.28
Safrole	-4.59	430.04	-5.65	72.86	-5.68	68.87	-6.39	20.60
Eugenol	-4.88	262.8	-6.07	35.49	-5.95	43.16	-7.19	5.35

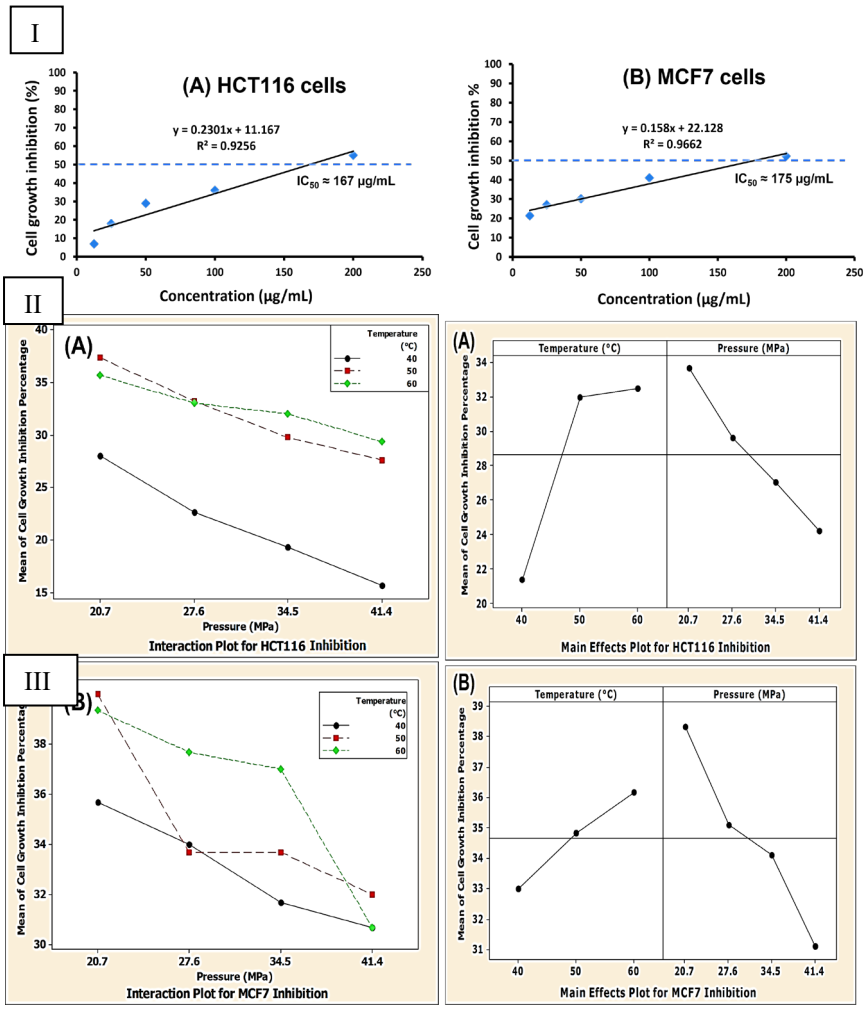

Figure 1 (I). Concentration-dependent effect of supercritical extracted oil of nutmeg seeds prepared at 50°C, 20.7 MPa on the cell viability of HCT116 and MCF7 cells. **(II).** The interaction effect of temperature and pressure on the HCT116 and MCF7 cell growth -inhibition. A; shows the interaction behavior of the pressure and temperature on the growth inhibition of HCT116 cells using nutmeg seed SFE extract. B; shows the interaction behavior of the pressure and temperature on the growth inhibition of MCF7 cells using nutmeg seed SFE extract. **(III).** Effects of pressure and temperature independently on the inhibition properties of the nutmeg SF extract. A: shows the effect of pressure (20.7, 27.6, 34.5, 41.4) or temperature (40, 50, and 60) on the inhibitory property of nutmeg SF extract on HCT 116 cells using. B: shows the influence of pressure or temperature on the inhibitory property of nutmeg SF extract on MCF7 cells using.

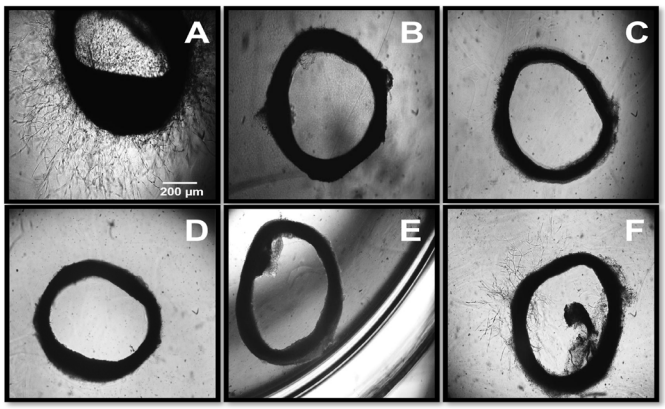

Figure 2. The anti-angiogenic effects of different extracts of nutmeg seed on the ex vivo rat aorta ring assay: The representative images shown were taken using an inverted phase-contrast microscope at 4× magnifications with the scale indicating 200 µm. The rat aorta rings treated with (A) DMSO showed a full growth of sprouting blood vessels from the ring (negative control), (B) Suramin (positive control), (C), (D), and (E) 200 µg/mL of the supercritical fluid extracts of nutmeg seeds at temperatures of 40, 50 and 60°C (all extracted with the pressures of 20.7 MPa), respectively, and (F) 200 µg/mL of Soxhlet extract of nutmeg seeds

3.6 Angiogenic effect of Nutmeg Seed SF Extract

The most potent nutmeg seed SFE extract against HCT116, and MCF7 cancer cell lines was selected to assess its antiangiogenic dose-dependent activity. This sample was extracted at an extraction pressure of 20.7 MPa, temperature of 50°C, using a CO2 flow rate of 1 mL/min and during 90 min of dynamic extraction time. The result showed that a dose-dependent inhibition was observed with 44, 57, 74, 100, 100 and 10031 µg/mL, respectively. The concentration of treatment (extract) which inhibits 50% of blood vessels' growth (IC50) was calculated using a linear regression equation, as shown in **Fig. 3a**. The IC50 for this extract on the angiogenesis of rat aortic ring was found to be 31 µg/mL. **Fig. 3b** shows the images of the dose-response inhibition of blood vessel formation using SFE of the nutmeg seeds.

3.7 Docking study analysis

The docking results of the VEGFA, COX1, EGF, and HIF structures with each ligand of 5FU and tamoxifen demonstrated the presence of numerous possible potential interactions were present (Fig. 4a, b, c, d). Myristicin, when interacting with VEGFA, established two hydrogen bonds with ASP63 and LYS107 (Fig. 4a). The chemical interactions between the EGF protein and the studied compounds (ligands) based on the docking studies are represented in Fig. 4b. The chemical interactions between the compounds and COX1 are represented in Fig. 6c. The chemical interactions between the compounds and the HIF protein are shown in Fig. 4d. Based on the obtained finding, myristicin showed a stronger affinity towards COX1, HIF and EGF molecules compared with 5FU, which is recognized as a therapeutic agent in colon cancer. However, tamoxifen, which was studied as a positive control in breast cancer, exhibited higher affinity to these three protein targets than 5FU and myristicin. The higher negative value of FBE, the better- important initial indicator of drug potency. On the other hand, other major compounds present in the nutmeg extract, such as α-asarone, safrole and eugenol, interacted with different proteins, as demonstrated in Table 4.

4. Discussion

Three out of 13 extracted samples were found to exhibit the highest inhibition effect on HCT116 and MCF7 cancer cells. These three samples were the SF extracted samples using 20.7 MPa of extraction pressure, extraction temperatures of 40°C, 50°C and 60°C, CO2 flow rate of 1 mL/min, sample size of <0.5 mm, and 90 min dynamic extraction time. It is worth noting that all nutmeg seed SFE extracts in this study demonstrated a significantly stronger inhibitory effect on HCT116 cells than on MCF7 cells. This is due to the fact that the MCF7 cell line has higher drug-resistant property than other cancer cell types. In fact, it has been reported that MCF7 cells exhibited less drug sensitivity than other human cancer cell lines toward the use of many extracts (Zu, et al., 2010).

Additionally, it is important to note that the supercritical extracts displayed markedly higher inhibitory activity than Soxhlet. This result can be confidently attributed to the presence of some active compounds in the nutmeg seed extracts, which is credited to the SFE extraction condition. The optimal extraction temperature in SFE plays a pivotal role in preserving the chemical composites of the extract and preventing their degradation, thereby surpassing the Soxhlet heat or distillation method (Ibrahim & Al-Rawi, 2018). This insight highlights the potential of using SFE as a more effective method for extracting bioactive compounds. Notably, the SFE low temperature has the ability to extract thermolabile compounds such as flavonoids, antioxidants and secondary metabolites and other bioactive volatile compounds (Xiao, et al., 2007; Ibrahim, et al., 2011). Moreover, reducing the fluid density by decreasing the extraction pressure aids in dissolving more compounds and increasing their solubility, which can then be recovered from the fluid (Ab Rahman, et al., 2010). In this study, GC/TOF-MS was used for the identification and characterization of the chemical compounds of nutmeg. In fact, many techniques proved their efficiency in the identification of chemical compounds such as X rays, NMR, FTIR and MALDITOF (Kamal, et al., 2023; Rudyk et al., 2023). However, GCTOFMS has a higher sensitivity in the identification of chemical composition of a mixture than other method. In addition, GCTOFMS has a higher efficiency in identifying the overlapped peaks and identifying compounds at the lowest concentration using small amount of sample (Ibrahim & Al-Rawi, 2018). The GCTOFMS analysis of SFE nutmeg extracts confirmed the presence of several active compounds from the aromatic ether group concentration, such as myristicin, eugenol, safrole, α-asarone, methyleugenol and many other compounds. Our result was in line with previous study, where the presence of these compounds was detected in nutmeg (Usui, et al., 2023). Myristicin, one of the major polyphenol compounds in nutmeg, was found at a higher concentration in all our nutmeg seed SFE extracts. In previous published study, myristicin induced cytotoxicity on human neuroblastoma SK-N-SH cells by an apoptotic mechanism (Lee, et al., 2009). Myristicin was reported to have anticancer properties and is known to be cancer chemopreventive agent of some medicinal plants (Seneme, et al., 2021). Additionally, our GCTOFMS results conclusively confirms the existence of various other compounds in the nutmeg SFE extract, including safrole, methyl eugenol, α-asarone, and α-thujene, which significantly augment the inhibitory effect. These potent compounds work together synergistically, creating an unbeatable effective formula. At a concentration of 10 µM, methyleugenol has been reported as a breast cancer invasion inhibitor and was cytostatic against breast

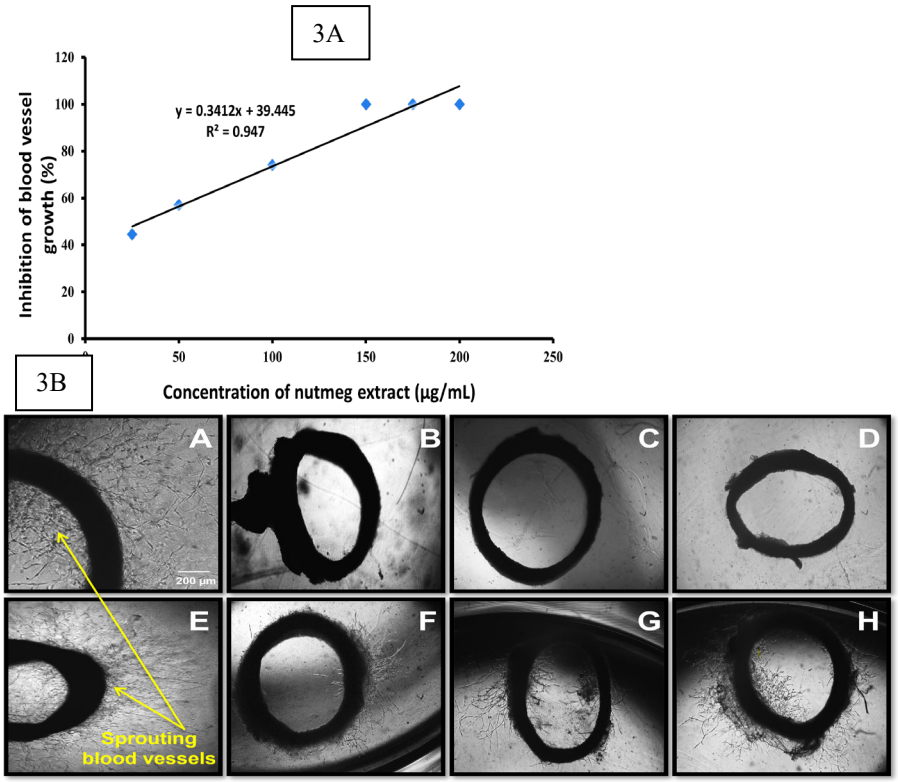

Figure 3. The anti-angiogenesis properties of nutmeg seed extracts. **A;** The dose-response effect of the supercritical fluid extract of nutmeg seeds at temperature of 50 °C and pressure of 20.7 MPa on the rat aorta vessel growth. The rings were treated using six serial concentrations of with the most active extract of nutmeg seed, with six replicates for each concentration. The IC50 of the nutmeg seed SFE was 31 µg/mL. **B.** the result of dose-response antiangiogenic effect of the SFE of nutmeg seeds on rat aorta rings was assessed by rat aorta ring assay. A & E; images of rat aorta rings treated with DMSO (0.1%) which show a full growth of blood vessel. B, C, D, F, G, and H: images of rings treated with 200, 175, 150, 100, 50 and 25 µg/mL of nutmeg SF extracts, respectively. The representative images were taken using an inverted phase-contrast microscope at 4X magnifications.

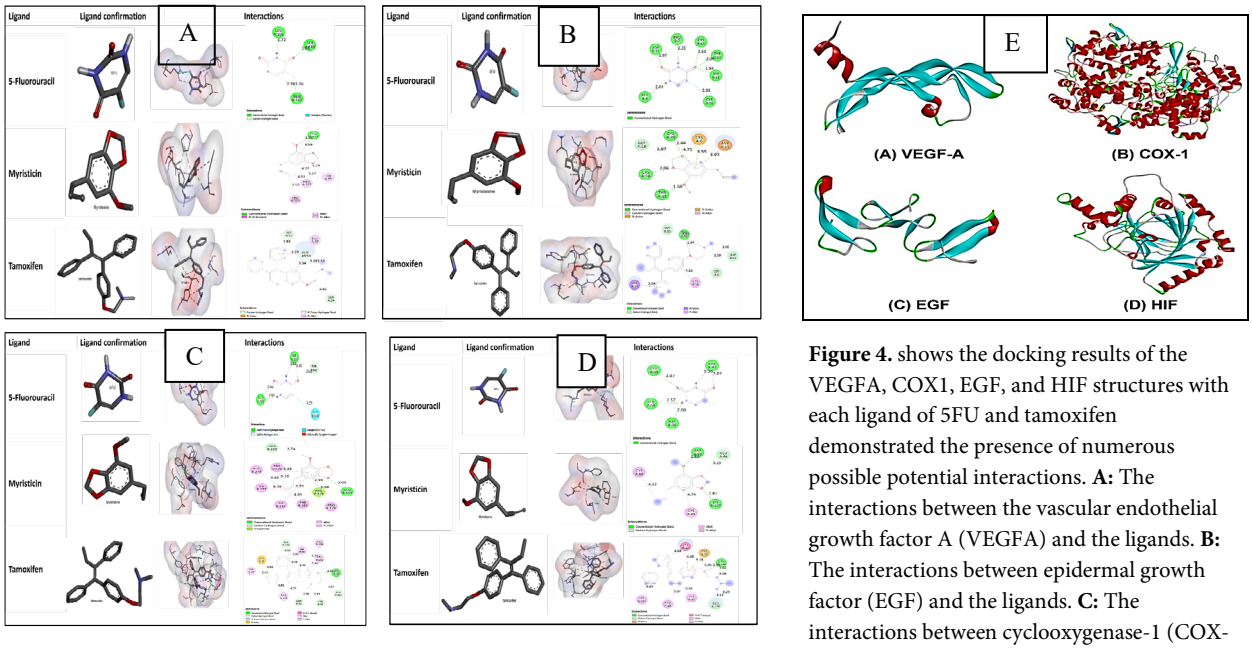

Figure 4. shows the docking results of the VEGFA, COX1, EGF, and HIF structures with each ligand of 5FU and tamoxifen demonstrated the presence of numerous possible potential interactions. **A:** The interactions between the vascular endothelial growth factor A (VEGFA) and the ligands. **B:** The interactions between epidermal growth factor (EGF) and the ligands. **C:** The interactions between cyclooxygenase-1 (COX-1) and the ligands. **D:** The interactions between hypoxia-inducible factor (HIF)-1 and the ligands. **E:** The protein structures from the RCSB protein data bank for (A) VEGF-A (ID: 4KZN), (B) COX-1 (ID: 1EQH), (C) EGF (ID:

cancer more than colchicine, the positive control (Bar, et al., 2010). Also, methyleugenol and safrole were reported to have a potent genotoxic effect with a DNA-binding potency (Barceloux, 2009). Likewise, α-thujene, a compound in nutmeg extract with many applications as folk medicine, is considered as one of the most notorious monoterpenes. In addition, the mode of action of α-thujene and the basis of its toxicity in humans have been proven by previous work (Crozier, et al., 2006). Moreover, the inhibitory effect of the extracted sample at 50°C extraction temperature dropped sharply with increasing the extraction pressure. Moreover, the inhibitory effect of the nutmeg seed SFE-extracted samples at temperatures of 40°C and 60°C were less than the effect of the extraction sample at 50°C. However, with pressure increment, the inhibitory effect of the nutmeg seed SFE-extracted sample at 40°C followed the same fashion but dropped a tad using 60°C of extraction temperature. This tendency could be due to the components present in the SFE extracts at 40 and 50°C. Yet, their concentrations differed in those two extracts, as the concentration of some compounds present in the extract at 50°C increased, giving it a potent effect. On the other hand, increasing the extraction pressure lessened the extract selectivity as a higher number of compounds were extracted. Additionally, the concentrations of several compounds were found to be less in the nutmeg seed SFE extracts at 60°C. This reveals the reason behind the different inhibitory effects of these extracts. Moreover, the combined effects of the various chemical ingredients contained in a single extract contribute to its quality, and certain active chemicals can be amplified or suppressed by another molecule present in the same extract (Ibrahim & Al-Rawi, 2018).

In the same fashion, the result confirms that the interaction between pressure and temperature resulted in a significant inhibitory effect on cell viability (P-value of 0.019). In fact, the inhibitory effect was reduced slightly by raising up the pressure from 20.7 MPa up to 27.6 MPa whereas the inhibitory effect of the nutmeg SFE extract was nearly the same when the pressure was increased to 34.5 MPa. The inhibitory effect of the nutmeg SFE extract significantly decreased when the pressure increased to 41.4 MPa, as revealed by the drop in the cell growth inhibition percentage. This could be due to the dropping of the SFE method selectivity at this operating pressure. In addition, increasing the extraction pressure improved the compounds' solubility in the extract due to the increase in fluid solvating influence of CO2 (Ibrahim, et al., 2017). The presence of these extra compounds may affect and counter the synergistic effect of each compound, which results in lowering the inhibitory effect. On the other hand, the increment in extraction temperature plays a major role in increasing the extracts' inhibitory effects on cancer cell growth when the pressure was fixed. In fact, raising up the extraction temperature from 40°C to 60°C increased the inhibition extract effect on cell growth gradually in a linear fashion. In contrast, increasing the extraction pressure did not follow the same trend in inhibiting the cell growth, instead, reducing the pressure increased the inhibition effect. This differential impact with the pressure variations might likely be attributed to a specific mixture of chemicals that can be extracted at a certain extraction condition and the subsequent dissolubility of these compounds at the end of the process (Ab Rahman, et al., 2012).

On the other hand, to investigate the antiangiogenic properties of the nutmeg seed extract, the ex vivo rat aortic ring assay was used to assess the outgrown blood vessels' length. This is a common, well-known assay to investigate angiogenesis (formation of blood vessels) in whole or partial organ cultures (Al-Rawi, et al., 2011). This assay is considered as a simple, rapid method, where the estimation of the antiangiogenic potential of a compound can be performed by quantifying the number and length of outgrown microvessels from the primary ex-plant (Ibrahim, et al., 2017; Al-Rawi, et al., 2011). Likewise, SFE extracts of nutmeg seeds inhibited all the new blood vessels whereas the Soxhlet extract of nutmeg seeds inhibited only 70% of the newly formed blood vessels. This outcome can be attributable to the advantageous circumstances of the supercritical extraction method, which allow for low-temperature extraction that helps to preserve heat-sensitive bioactive chemicals and keeps them from volatilizing at higher temperatures. Additionally, the supercritical extraction method uses carbon dioxide, which is thought to be a good medium for extracting volatile chemicals under low pressure (King, 2002). SFE is well known to be an innovative extraction technique that may provide the maximum yield of chemicals (Chen, et al., 2010). In addition, polyphenols compounds, one of the major components of nutmeg, have a pronounced influence on cancer angiogenesis and have the ability to block the angiogenesis process by inhibiting the formation of blood vessels (Kim, 2003). In addition, it is worth mentioning that numerous epidemiological studies have suggested that polyphenols have chemopreventive properties. It has been demonstrated that phenolic compounds have strong antioxidant properties, decrease endothelial cell angiogenesis, and restrain the growth of tumor cells in vitro (Diniz, et al., 2017). It's crucial to remember, though, that our chemical analysis of the nutmeg seed SFE extracts revealed the presence of several polyphenols, including eugenol and its derivatives. The inclusion of terpenoids such as α-terpinene, α-phellandrene, and α-cubebene as well as other chemicals like safrole, elemicin, myristicin, and α-asarone may potentially have an impact on the action of this extract due to the synergistic effect between them. However, to investigate the dose-dependent angiogenic effect of nutmeg SF extracts, the most potent nutmeg seed SFE extract against HCT116, and MCF7 cancer cell lines was selected. This sample was extracted at an

extraction pressure of 20.7 MPa, temperature of 50°C, using a CO_2 flow rate of 1 mL/min and during 90 min of dynamic extraction time. The IC50 for this extract on the angiogenesis of rat aortic ring was found to be significant with a 31 µg/mL. The anti-angiogenic potential of SFE nutmeg seed extract is most likely due to the presence of myristicin and other potent compounds. The GC/TOF-MS result showed that the nutmeg SFE which was extracted at an extraction pressure of 20.7 MPa and temperature of 50°C had a higher concentration of myristicin compared to other nutmeg SFE extracts which was published previously (Ibrahim & Al-Rawi, 2018). This suggests the important role of myristicin in the inhibition of angiogenesis. The role of myristicin and eugenol have been attributed due to their protective characteristic in inhibiting the inflammatory cytokine, tumor necrosis factor (TNF)-α, from the macrophages (Miller & Ruiz-Larrea, (2002; Jaiswal et al., 2009). It's interesting to note that TNF-, a potent marker of inflammatory disorders like rheumatoid arthritis can promote the hyperproliferation that occurs during carcinogenesis (Aggarwal, et al., 2009; Jaiswal et al., 2009). This finding could explain why traditional medicine has successfully treated rheumatism with nutmeg oil. Utilizing nutmeg extract to treat rheumatism may be helpful due to its effectiveness in inhibiting the excessive angiogenesis, which is the pathological cause of rheumatism. These findings unequivocally establish nutmeg extract as an assertive and potent natural inhibitor of specific biological processes.

Molecular docking was conducted to understand the molecular interaction mechanism of the main active compounds of nutmeg seed extract with the molecular mediators involved in angiogenesis (COX-1, VEGFA, HIF, and EGF). A comprehensive mechanism to assess the binding of the main active compound with molecular mediators involved with angiogenesis is achievable by the structure-activity prediction using the molecular docking approach (Murray & Pizzorno, (2010). The molecular docking results of our study showed that the principal compounds of nutmeg, as myristicin, displayed strong interactions with angiogenesis molecular mediators; COX-1, VEGFA, HIF, and EGF compared with tamoxifen and 5-fluorouracil (5-FU). Myristicin, when interacting with VEGFA, established two hydrogen bonds with ASP63 and LYS107 (Fig. 6a). A carbon-hydrogen bond was also established with GLU64 and 2 alkyl bonds with CYS68 and CYS61 with free binding energy of -4.57 Kcal/mol. Tamoxifen, a positive breast cancer control, was used for comparison, displayed one hydrogen bond with CYS104 and CYS26 with pi sulfur bond. Also, a pi T-shaped bond was present coupled with TYR25 and three alkyl bonds, LYS101, PRO28 and HIS27. In addition, a carbon-hydrogen bond with GLU103 was shown with -7.22 Kcal/mol free binding energy. The 5FU, which was selected as another positive breast cancer control, after interaction with VEGFA, showed four hydrogen bonds with PHE47, PHE36, SER50 and ASP34 with the free binding energy of -6.71 Kcal/mol. The chemical interactions between the EGF protein and the studied compounds (ligands) based on the docking studies are represented in Fig. 6b. Myristicin showed H bonds with CYS20, CYS14 and TYR13. It also displayed a pi sulfur bond with CYS6 and ASP11 and a carbon-hydrogen bond with GLY18 with -5.7 Kcal/mol of free binding energy. Tamoxifen showed a hydrogen bond with TYR17 and a pi sigma bond with VAL19. It showed an alkyl bond with CYS14 and three carbon-hydrogen bonds with LEU8, ASP11 and CYS20 with free binding energy of -6.58 Kcal/mol. Although 5FU involves six predictable H bonds via LEU8, CYS14, ASP11, PRO7, GLY12, TYR13 and CYS14. All the synthesized ligands showed a stronger affinity towards EGF protein compared with it; this may be due to the stability of the binding pocket 38. Among all the studied compounds, tamoxifen demonstrated the lowest free binding energy and the highest tendency to the EGF molecule. After that, myristicin showed higher affinity towards EGF compared with 5FU. Myristicin causes more stability in bonding to the active pocket of the molecule, which causes it to be more stable. The chemical interactions between the compounds and COX1 are represented in Fig. 6c. Myristicin demonstrated a hydrogen bond with ASN375 and a pi lone pair with ARG376. It showed a carbon-hydrogen bond with ARG150 and six alkyl bonds with PHE529, ALA378, ILE377, ILE124, PHE381 and PRO128 with -7.2 Kcal/mol of free binding energy. Tamoxifen showed a hydrogen bond with HIS43 and carbon-hydrogen bonds with GLU465, TYR39 and GLN42. It also displayed a pi sulphur bond with CYS41 and five alkyl bonds with PRO153, CYS47, ILE46, ARG469 and LEU152 with -10.18 Kcal/mol of free binding energy. 5FU showed H bonds with ILE137 and THR331, and a halogen bond with SER548, and the presence of fluorine in ligand structure. Though, the free binding energy of tamoxifen was less than 5FU and myristicin. The chemical interactions between the compounds and the HIF protein are shown in Fig. 6d. Myristicin also showed an H bond with PHE244 and three alkyl bonds, LYS99, PRO197 and LEU101 with -5.82 Kcal/mol of free binding energy. Tamoxifen showed a carbon-hydrogen bond with SER13, GLU29 and SER34. It showed a pi anion bond with GLU29 with -7.57 Kcal/mol free binding energy. 5FU displayed three H bonds: LEU101, SER118 and GLN147. Moreover, it displayed a halogen bond between the fluorine atom in 5FU with GLN147 with -5.26 Kcal/mol of free binding energy.

In addition to myristicin, the molecular docking results of our study showed that the other investigate compounds of nutmeg, α-asarone, safrole and eugenol displayed very strong interactions with angiogenesis molecular mediators; COX-1, VEGFA, HIF, and EGF compared with tamoxifen and 5-fluorouracil (5-FU). These

compounds interacted potentially with cell growth factors COX1, HIF and EGF molecules in the same fashion as myristicin. This result was in line with previous result from where nutmeg oil inhibited COX-2 expression and alleviated chronic inflammatory pain in an in-vivo study Zhang, et al., (2016). The results of free binding energy for each of these compounds showed their potential effectiveness in treating cancer. However, based on our results, it can be suggested that among these compounds, myristicin and eugenol have more potential power to be developed as a therapeutic agent against colon cancer. Interestingly, this trend is different with regard to the inhibition of VEGFA, where tamoxifen showed the strongest affinity towards inhibition of VEGFA. However, 5FU showed lower free binding energy than myristicin which determines 5FU as a stronger ligand to VEGFA than myristicin. Even though tamoxifen showed lower free binding energy than myristicin and makes it a stronger agent inhibiting VEGFA, it is known to cause severe side effects; in comparison, myristicin could be less toxic being obtained from natural extracts. Typically, chemotherapy uses cytotoxic chemicals to stop the growth of cancer cells. However, normal cells with a high proliferative index have significant adverse effects from these medications. Therefore, there is a huge demand for innovative therapies with enhanced characteristics. Natural angiogenic inhibitors have recently emerged as a possible tumor therapy (Mukund, et al., 2019). Recent study addressed the use of nutraceuticals in treating angiogenesis-dependent disorders (Morbidelli, et al., 2018). These natural compounds have shown prominent anti-angiogenic effects in the preclinical models of tumor angiogenesis against various malignancies (Shanmugam, et al., 2017). Growth factors and receptors like VEGF/VEGFR, bFGF/FGFR, angiopoietins, and hypoxia-inducible factors promote angiogenesis and can be targeted for anti-cancer treatment (Li, et al., 2023; Liu, et al., 2023). Semi-synthetic derivatives and nano-formulations of these natural compounds have shown promising results by improving drug delivery and bioavailability (Elmowafy, et al., 2023; Kumar et al., 2023). Moreover, some of these compounds were found to inhibit cox (Zhang, et al., 2016).

Our experimental results of the molecular modelling study (interaction with VEGFA), suggest that myristicin, α-asarone, safrole and eugenol that present in the nutmeg seed extracts might contribute to the antiangiogenic and antiproliferative effects of the tested extracts and could be behind the ex vivo rat aorta ring results. However, nutmeg oil contains many other active compounds with anticancer and antiangiogenic effects that need to be further investigated. It is also possible that other compounds present in the nutmeg seed extract have overlapping effects and might cause different cumulative efficacy. So, further toxicity and in vivo studies are recommended to identify the efficacy of such natural compounds in inhibiting colon and breast cancer.

5. Conclusion

This study emphasizes the immense potential of nutmeg extract as a potent and reliable natural inhibitor of specific biological processes. The nutmeg seed extract has been found to contain several active compounds that possess significant medicinal properties. Myristicin was identified as the main constituent of the extract, with a high quantity in the SFE of nutmeg seeds. The extract has demonstrated potential inhibitory activity against the formation of new blood vessels and the proliferation of cancer cells. Molecular docking results have shown that myristicin, α-asarone, safrole, and eugenol have effective potentially binding interactions to inhibit all the cancer-related proteins and angiogenesis molecular mediators. Other compounds present in the nutmeg extract, so it is plausible that those compounds have overlapping or synergistic effects to exert a cumulative outcome of anticancer and antiangiogenic property. Further research should focus on evaluating the individual compounds present in the extract to explore its full potential and develop promising therapies from nutmeg seeds.

Author Contributions

Conceptualization, SSA, and AHI; methodology, SSA, and AHI; software, MZ, and MAH.; visualization, SSA, DB; formal analysis, SSA, AHI, MAH; investigation, SSA, AHI; writing original draft & preparation, SSA, AHI; review and editing, SSA, HAE and DB; supervision, MOA, ASAM, AMSA.

Acknowledgment

The authors acknowledged the university Science Malaysia for all the provided materials.

Competing financial interests

The authors have no conflict of interest.

References

Ab Rahman, N. N. N., Nama, M. M. B., Al-Rawi, S. S., Ibrahim, A. H., & Ab Kadir, M. O. (2011). Comparison of nutritional composition between palm kernel fibre and the effect of the supercritical fluid extraction on its quality. Procedia Food Science, 1, 1940-1945.

Ab Rahman, N. N., Al-Rawi, S. S., Ibrahim, A. H., Nama, M. M. B., & Ab Kadir, M. O. (2012). Supercritical carbon dioxide extraction of the residual oil from palm kernel cake. Journal of food engineering, 108(1), 166-170.

Aggarwal, B. B., Van Kuiken, M. E., Iyer, L. H., Harikumar, K. B., & Sung, B. (2009). Molecular targets of nutraceuticals derived from dietary spices: potential role in suppression of inflammation and tumorigenesis. Experimental Biology and Medicine, 234(8), 825-849.

Al-Ostoot FH, Salah S, Khamees HA, Khanum SA. (2021). Tumor angiogenesis: Current challenges and therapeutic opportunities. Cancer Treat Res Commun, 28, 100422

Al-Rawi, S. S., Ibrahim, A. H., Ab Rahman, N. N. N., Nama, M. M. B., Majid, A. M. A., & Ab Kadir, M. O. (2011). The effect of supercritical fluid extraction parameters on the nutmeg oil extraction and its cytotoxic and antiangiogenic properties. Procedia Food Science, 1, 1946-1952.

Al-Rawi, S. S., Ibrahim, A. H., Majid, A. S. A., Majid, A. M. A., & Ab Kadir, M. O. (2013). Comparison of yields and quality of nutmeg butter obtained by extraction of nutmeg rind by soxhlet and supercritical carbon dioxide (SC-CO2). Journal of food engineering, 119(3), 595-601.

Baldo P, Fornasier G, Francescon S, Ferrarin E, Truccolo I, De Paoli P. (2015). Toxicities and adverse drug reactions experienced during anticancer treatment: it is desirable to consider the problem within the international system of pharmacovigilance. J Clin Oncol, 33(25), 2824–5

Bar, F. M. A., Khanfar, M. A., Elnagar, A. Y., Badria, F. A., Zaghloul, A. M., Ahmad, K. F., ... & El Sayed, K. A. (2010). Design and pharmacophore modeling of biaryl methyl eugenol analogs as breast cancer invasion inhibitors. Bioorganic & medicinal chemistry, 18(2), 496-507.

Barceloux, D. G. (2009). Nutmeg (Myristica fragrans Houtt.). Disease-a-Month, 6(55), 373-379.

Barman, R., Bora, P. K., Saikia, J., Kemprai, P., Saikia, S. P., Haldar, S., & Banik, D. (2021). Nutmegs and wild nutmegs: An update on ethnomedicines, phytochemicals, pharmacology, and toxicity of the Myristicaceae species. Phytotherapy Research, 35(9), 4632-4659.

Chen, W. H., Chen, C. H., Chang, C. M. J., Liau, B. C., & Hsiang, D. (2010). Supercritical carbon dioxide extraction of triglycerides from Aquilaria crassna seeds. Separation and Purification Technology, 73(2), 135-141.

Crozier, A., Clifford, M. N., & Ashihara, H. (2006). Plant secondary metabolites. Occurrence, Structure and Role in the Human Diet, Blackwell-Publishers.

Dahham, S. S., Al-Rawi, S. S., Ibrahim, A. H., Majid, A. S. A., & Majid, A. M. S. A. (2018). Antioxidant, anticancer, apoptosis properties and chemical composition of black truffle Terfezia claveryi. Saudi journal of biological sciences, 25(8), 1524-1534.

Diniz, C., Suliburska, J., & Ferreira, I. M. (2017). New insights into the antiangiogenic and proangiogenic properties of dietary polyphenols. Molecular nutrition & food research, 61(6), 1600912.

Diniz, C., Suliburska, J., & Ferreira, I. M. (2017). New insights into the antiangiogenic and proangiogenic properties of dietary polyphenols. Molecular nutrition & food research, 61(6), 1600912.

Elmowafy, M., Shalaby, K., Elkomy, M. H., Alsaidan, O. A., Gomaa, H. A., Abdelgawad, M. A., & Mostafa, E. M. (2023). Polymeric Nanoparticles for Delivery of Natural Bioactive Agents: Recent Advances and Challenges. Polymers, 15(5), 1123.

Ha, M. T., Vu, N. K., Tran, T. H., Kim, J. A., Woo, M. H., & Min, B. S. (2020). Phytochemical and pharmacological properties of Myristica fragrans Houtt.: an updated review. Archives of Pharmacal Research, 43, 1067-1092.

Habib, A., Nazari, M., Iqbal, M. A., Bhatti, H. N., Ahmed, M. K., & Majid, A. A. (2019). Unsymmetrically substituted benzimidazolium based Silver (I)-N-heterocyclic carbene complexes: Synthesis, characterization and in vitro anticancer study against human breast cancer and colon cancer. Journal of Saudi chemical society, 23(7), 795-808.

Hamdi, R. A., Al-Rawi, S. S., Ibrahim, A. H., & Kala, D. (2023). Potential Therapeutic and Effective Properties of Soft Tissue Liners. Journal of Population Therapeutics and Clinical Pharmacology, 30(4), 447-456.

Hanif, A., Ibrahim, A. H., Ismail, S., Al-Rawi, S. S., Ahmad, J. N., Hameed, M., ... & Tanwir, S. (2023). Cytotoxicity against A549 Human Lung Cancer Cell Line via the Mitochondrial Membrane Potential and Nuclear Condensation Effects of Nepeta paulsenii Briq., a Perennial Herb. Molecules, 28(6), 2812.

Ibrahim A, Al-Rawi S, Abdul Majid A, Al-Habib O, Abdul Majid A. (2013). Pro-angiogenic and wound healing potency of virgin coconut oil. Supp Care Cancer, MASCC. 21:235

Ibrahim, A. H., & Al-Rawi, S. S. (2018, December). Evaluating the supercritical extraction effects on the chemical compositions of the extracted oil using GC/TOF MS. In IOP Conference Series: Materials Science and Engineering (Vol. 454, No. 1, p. 012166). IOP Publishing.

Ibrahim, A. H., Al-Rawi, S. S., Majid, A. A., Rahman, N. A., Abo-Salah, K. M., & Ab Kadir, M. O. (2011). Separation and fractionation of Aquilaria malaccensis oil using supercritical fluid extraction and tthe cytotoxic properties of the extracted oil. Procedia Food Science, 1, 1953-1959.

Ibrahim, A. H., Khan, M. S. S., Al-Rawi, S. S., Ahamed, M. B. K., Majid, A. S. B. A., Al-Suede, F. S. R., ... & Majid, A. M. S. A. (2016). Safety assessment of widely used fermented virgin coconut oil (Cocos nucifera) in Malaysia: Chronic toxicity studies and SAR analysis of the active components. Regulatory Toxicology and Pharmacology, 81, 457-467.

Ibrahim, A. H., Li, H., Al-Rawi, S. S., Majid, A. S. A., Al-Habib, O. A., Xia, X., ... & Ji, D. (2017). Angiogenic and wound healing potency of fermented virgin coconut oil: in vitro and in vivo studies. American journal of translational research, 9(11), 4936.

Jaiswal, P., Kumar, P., Singh, V. K., & Singh, D. K. (2009). Biological effects of Myristica fragrans. Annual review of biomedical sciences, 11, 21-29.

Kamal, A., Ibrahim, A. H., Al-Rawi, S. S., Iqbal, M. A., & Bhatti, H. N. (2023). Biological potential of vinyl/allyl substituted imidazole-based N-heterocyclic carbene adducts: synthesis, spectral and X-ray crystallographic structural characteristics. Chemical Papers, 1-10.

Khan, T., Ali, M., Khan, A., Nisar, P., Jan, S. A., Afridi, S., & Shinwari, Z. K. (2019). Anticancer plants: A review of the active phytochemicals, applications in animal models, and regulatory aspects. Biomolecules, 10(1), 47.

Kim, M. H. (2003). Flavonoids inhibit VEGF/bFGF-induced angiogenesis in vitro by inhibiting the matrix-degrading proteases. Journal of cellular biochemistry, 89(3), 529-538.

King, J. W. (2002). Supercritical fluid extraction: present status and prospects. Grasas y Aceites, 53(1), 8-21.

Kumar, P., Mangla, B., Javed, S., Ahsan, W., Musyuni, P., Sivadasan, D., ... & Aggarwal, G. (2023). A review of nanomaterials from synthetic and natural molecules for prospective breast cancer nanotherapy. Frontiers in Pharmacology, 14, 1149554.

Lee, W., Patel, J. H., & Lockhart, A. C. (2009). Novel targets in esophageal and gastric cancer: beyond antiangiogenesis. Expert opinion on investigational drugs, 18(9), 1351-1364.

Li, X., Zhou, J., Wang, X., Li, C., Ma, Z., Wan, Q., & Peng, F. (2023). New advances in the research of clinical treatment and novel anticancer agents in tumor angiogenesis. Biomedicine & Pharmacotherapy, 163, 114806.

Liu, Z. L., Chen, H. H., Zheng, L. L., Sun, L. P., & Shi, L. (2023). Angiogenic signaling pathways and anti-angiogenic therapy for cancer. Signal Transduction and Targeted Therapy, 8(1), 198.

Lugano R, Ramachandran M, Dimberg A. (2020). Tumor angiogenesis: causes, consequences, challenges and opportunities. Cell Mol Life Sci, 77:1745–70,

Mattiuzzi C, Lippi G. (2019). Current cancer epidemiology. J Epidemiol Glob Health, 9(4), 217

Miller, N. J., & Ruiz-Larrea, M. B. (2002). Flavonoids and other plant phenols in the diet: Their significance as antioxidants. Journal of nutritional & environmental medicine, 12(1), 39-51.

Montemagno C, Pagès G. (2020). Resistance to anti-angiogenic therapies: a mechanism depending on the time of exposure to the drugs. Front Cell Dev Biol, 8, 584

Morbidelli, L., Terzuoli, E., & Donnini, S. (2018). Use of nutraceuticals in angiogenesis-dependent disorders. Molecules, 23(10), 2676.

Mukund, V., Saddala, M. S., Farran, B., Mannavarapu, M., Alam, A., & Nagaraju, G. P. (2019). Molecular docking studies of angiogenesis target protein HIF-1α and genistein in breast cancer. Gene, 701, 169-172.

Murray, M. T., & Pizzorno, J. (2010). The encyclopedia of healing foods. Simon and Schuster.

Nazari, M., Mahmood, S., & Raman, S. (2019). Comparative docking studies of rosmarinic acid and sinesitin to inhibit HSP70. International Journal of Engineering Technology and Sciences, 6(1), 115-119.

Nazari, M., Nazari, M., & Arabani, S. (2021). Anti-Inflammation Prediction of Orthosiphon Stamineus Extract Against Covid19 (In Silico Study). International Journal of Engineering Technology and Sciences, 8(1), 14-18.

Rudyk, S., Ongarbayev, Y., & Spirov, P. (2023). Feature selection in GC-MS, NMR and MALDI-TOF spectra of tar sand bitumen. Unconventional Resources, 3, 61-71.

Rust R. (2020). Insights into the dual role of angiogenesis following stroke. J Cereb Blood Flow Metab, 40(6), 1167–71.

S Al-Rawi, S., H Ibrahim, A., & B Ahmed, G. (2023). Unraveling The Link Between Lymphopenia And Systemic Lupus Erythematosus: Implications For Disease Severity And Potential Treatment Strategies. Eurasian Journal of Science and Engineering, 9(2).

Seneme, E. F., Dos Santos, D. C., Silva, E. M. R., Franco, Y. E. M., & Longato, G. B. (2021). Pharmacological and therapeutic potential of myristicin: A literature review. Molecules, 26(19), 5914.

Shanmugam, M. K., Warrier, S., Kumar, A. P., Sethi, G., & Arfuso, F. (2017). Potential Role of Natural Compounds as Anti-Angiogenic Agents in Cancer. Current vascular pharmacology, 15(6), 503-519.

Sim, H. W., Wachsmuth, L., Barnes, E. H., Yip, S., Koh, E. S., Hall, M., ... & Khasraw, M. (2023). NUTMEG: A randomized phase II study of nivolumab and temozolomide versus temozolomide alone in newly diagnosed older patients with glioblastoma. Neuro-Oncology Advances, 5(1), vdad124.

Usui, K., Kubota, E., Kobayashi, H., Fujita, Y., Hatanaka, K., Kamijo, Y., ... & Mimasaka, S. (2023). Detection of major psychoactive compounds (safrole, myristicin, and elemicin) of nutmeg in human serum via GC–MS/MS using MonoSpin® extraction: Application in a nutmeg poisoning case. Journal of Pharmaceutical and Biomedical Analysis, 234, 115565.

Wang H, Oo Khor T, Shu L, Su ZY, Fuentes F, Lee JH, et al. (2012). Plants vs. cancer: a review on natural phytochemicals in preventing and treating cancers and their drug ability. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 12(10), 1281-1305.

Xiao, J. B., Chen, J. W., & Xu, M. (2007). Supercritical fluid CO2 extraction of essential oil from Marchantia convoluta: global yields and extract chemical composition. Electronic Journal of Biotechnology, 10(1), 141-148.

Yang, T., Xiao, H., Liu, X., Wang, Z., Zhang, Q., Wei, N., & Guo, X. (2021). Vascular normalization: a new window opened for cancer therapies. Frontiers in Oncology, 11, 719836.

Zhang, W. K., Tao, S. S., Li, T. T., Li, Y. S., Li, X. J., Tang, H. B., ... & Wan, C. J. (2016). Nutmeg oil alleviates chronic inflammatory pain through inhibition of COX-2 expression and substance P release in vivo. Food & nutrition research, 60(1), 30849.

Zu, Y., Yu, H., Liang, L., Fu, Y., Efferth, T., Liu, X., & Wu, N. (2010). Activities of ten essential oils towards Propionibacterium acnes and PC-3, A-549 and MCF-7 cancer cells. Molecules (Basel, Switzerland), 15(5), 3200–3210. https://doi.org/10.3390/molecules15053200.