SciVerse ScienceDirect

Procedia Food Science 1 (2011) 1946 – 1952

Procedia
Food Science

11th International Congress on Engineering and Food (ICEF11)

The effect of supercritical fluid extraction parameters on the nutmeg oil extraction and its cytotoxic and antiangiogenic properties

Sawsan S. Al-Rawi[a], Ahmad H. Ibrahim[b], Nik Norulaini Nik Ab Rahman[c] *, Moftah M. Ben Nama[c], Amin M. S. Abdul Majid[b], Mohd Omar Ab Kadir[a]

[a] *School of Industrial Technology, Universiti Sains Malaysia,Penang 11800, Malaysia*
[b] *EMAN Testing & Research Laboratory, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang, 11800, Malaysia*
[c] *School of Distance Education, Universiti Sains Malaysia, Penang 11800, Malaysia*

Abstract

In this study, the influence of supercritical extraction parameter on the oil extraction of Nutmeg (*Myristica fragrans)* was investigated, and then the extracted oil was tested for its cytotoxic and anti-angiogenic activity. Supercritical extraction was carried out using the operation pressures 20.7, 27.6, 34.5 and 41.4 MPa, and temperature was varied between 40 and 50°C. The CO_2 flow rate was between 1-3 ml/min using matrix particle size ≤ 1mm and during extraction time of 90 min. The extracted oils were tested for anti-angiogenic activity using a 3 dimensional ex-vivo isolated rat aorta tissue. MTT assay was used as *in vitro* study to investigate the cytotoxic properties of the extracted oil samples on two types of human tumour cell lines, colon cancer cell HCT-116 and breast cancer cell MCF7. The study reveal that the extraction yield depends on pressure and temperature, and there is a significant difference between temperatures 50°C and 40°C at different pressure, whereby increasing pressure leads to increase extraction yields significantly. However, varying the flow rate has no effect on the extraction yield. The anti-angiogenic inhibitions were significant at concentration of 200 µg /ml nutmeg oil. MTT assay results indicate that all the extracts under different extraction parameters are noncytotoxic. These results confirmed that supercritical extraction could be a promising technique to produce high quality of botanicals extracts, free of solvent, noncytotoxic with significant cost savings.

Selection and/or peer-review under responsibility of 11th International Congress on Engineering and Food (ICEF 11) Executive Committee.

Keyword: Nutmeg; supercritical; extraction; Anti-angiogenesis; cytotoxicity

* Corresponding author. Tel.: +60 46585435; fax: +60 46585435.
E-mail address: norulain@usm.my.

doi:10.1016/j.profoo.2011.09.286

1. Introduction

Myristica fragrans is a tropical, ever green dioeciously tree and commonly available in Penang, Malaysia, India, Indonesia and South East of Asia. Nutmeg is the dried kernel of the seed of *Myristica fragrans*. It has a pleasant aroma and warm taste and it is used as a spice in a large number of countries. Its extract consists of fixed oil, volatile and essential oil with different concentration. In the industries, it has been used as a natural flavoring in beverages, candies, syrups and perfumes. In medicine, it has been used externally as dental creams, a pain relieving ointment, relieving sprains and rheumatism. It was found that it has aphrodisiac, stomachic, anti-fungal, nerve stimulant, antithrombotic, anti-dysenteric, anti-inflammatory and hepatoprotective effects [1].

Angiogenesis is the formation of new blood vessels from preexisting one. This process occurs essentially during the embryonic development, reproduction and wound healing while it is pathological during the tumor growth, providing tumor cells with vital nutrients to promote it growth. Blocking these blood vessels by anti-angiogenic agent could suppress tumor growth. It was found that tumor treatment combining angiogenic inhibitors with chemotherapy drugs and radiation therapy, works more effectively [2]. Edris [3] clarified that most cancer chemotherapy procedure uses cytotoxic drugs to target the proliferating cell populations. However, these drugs have severe side effects on normal cells with a high proliferative index, thus the use of natural angiogenic inhibitors offer a promising treatment for tumor [4].

Drug safety is still a major concern for researchers and also for the pharmaceutical companies. Traditional extraction methods have some typical disadvantages which include high costs, long analysis period, low samples throughput and the need for high qualified manpower plus the residual solvent. Thus a clean, fast, reliable and simple extraction method is a target to improve the production. Therefore, this paper proposes employing rapid and safe extraction method for pharmaceutical industry. Supercritical carbon dioxide (SC-CO_2) which is a cleaner and safer alternative to other traditional extraction methods can be applied in pharmaceuticals in the production of natural products free of residual solvent. Furthermore, employing carbon dioxide as a supercritical solvent is a promising technology. It does not affect thermally sensitive materials with it low critical temperature (31.1°C) and pressure (7.28 MPa), that makes it an ideal solvent for extracting. Also being nontoxic, inflammable, widely available and cheap [5]. Therefore, the aim of this study is to investigate the supercritical fluid extraction parameters that affect the yield of the nutmeg oil as well as investigating the angiogenic activity and cytotoxicity of the nutmeg extract on human cancer cells.

2. Materials & Methods

2.1. Materials and chemicals

Commercial liquid carbon dioxide gas with purity of 99 g kg^{-1} was purchased locally from Malaysian Oxygen, Penang, Malaysia in a gas cylinder at temperature below -5ºC. Nutmeg samples were purchased from a farm in Balik Pulau, Penang, Malaysia. Human cancer cell line was purchased from Sciencecell, USA. Cell culture media RPMI 1640, foetal bovine serum (FBS), Pencilling and Streptomycin solutions were from Gibco, USA. MTT cell proliferation assay kit was from Sigma Aldrich, Germany. The rat aorta ring assay reagents were purchased from Sigma Aldrich, Germany. The HCT-116 and MCF7 cell line were maintained in RPMI 1640 cell culture medium supplemented with 10% FBS and 1% P/S. The cells were cultured in class II biosafety cabinet (ESCO, USA) under sterile conditions.

2.2. Samples preparation & oil extraction

The samples were washed then dried in the oven at 50°C for six h. The nutmeg samples were grinded using analytical mill model IKA(R) A11 from Retsch, and were sieved to obtain a matrix particle size ≤ 1

mm. The samples were kept in zipper polypropylene bags at -20°C ready for extraction. A general factorial design with multi-level, base block one, was applied to get the highest extraction oil yield from the nutmeg samples. Two independent variables studied were extraction pressure (MPa) and temperature (°C). These independent variables and their levels were selected based on the preliminary experiments in our laboratory (data not shown). Response (the oil yield) at each design point was recorded, and each extraction was run in three replicates. Supercritical CO2 extractions were performed using the extraction system consists of (ISCO, Inc., Lincoln, NE. USA model SFX 220). The extraction was run with 1.5 g of sample placed in the extraction vessel for extraction. The oil was extracted at temperature of 40 and 50°C with operating pressure of 20.7, 27.6, 34.5 and 41.4 MPa using carbon dioxide flow rate 1 ml/min. At the end of the extraction, the supercritical fluid was depressurized to the atmosphere. The oil was collected in a pre-weighted collection glass vial. The oil yield was calculated by the weight increment at the end of the extraction and kept at -20°C ready for analysis.

2.3. Angiogenesis assay

The angiogenic inhibition was investigated by employing the rat aortic ring assay. The experiment was done according to the guide and the Permission from the Animal Ethics Committee of School of Pharmaceutical Sciences, Universiti Sains Malaysia**.** Adult male Sprague Dawley rats at age 8-10 weeks were received from the Animal House and allowed to acclimatize in the lab, where food and water were provided with changing the bedding every other day. The animals were sacrificed by cervical dislocation and the thoracic aortas were removed. The aortas were cleaned from the fibro-adipose tissue, and were cut into 1 mm long cross sections. The aortas rings were cultured in a 48-wells plate, each ring in one well, that were pre-coated earlier with 300µl of fresh culture media (M199) supplemented with fibrinogen 3mg/ml, approtinin 5 mg/ml and L-glutamine (1%). A 10 µl of thrombin was added to the each well. The plate was kept at 37°C for 60-90 min to solidify. Meanwhile, treatment were prepared in a final concentration of 200 µg/ml from supercritical extracted oil mixed with fresh growth media (M199) supplemented with 20% heat inactivated human bovine serum (HIFBS), 1% L-glutamine, 0.1% aminocaproic acid, 1% amphotericin B and 0.6% gentamicin and 1% fungizone. After incubation, 300 µl of the treatment was added to each well and was incubated again with 5% CO_2 at 37ºC for four d. Suramine as a positive control and dimethyl sulfoxide as a negative control at a concentration of 100 µg/ml was used too. Each sample was done in five replicates as well as the negative and positive control. At the day four, the upper layer was replaced with fresh growth media containing the drug in a concentration of 200 µg/ml. At day five, explants for micro-vessel outgrowths that occur at the cut surfaces of the aortic rings were examined and measured by a light microscope supplied with Leica Quin computerized imaging system, under a 4x magnification power. The experiment was repeated three times for validation.

2.4. Nutmeg cytotoxicity test

Cytotoxicity of nutmeg oil on tumor cells was measured by micro-culture tetrazolium (MTT) assay [6]. A 96-well microplate were seeded with 300 µl medium containing 1x 10^4 cells of Human colorectal carcinoma cell line (HCT 116) in suspension and incubated for at least 24 h prior to treatment. The cells were treated with 8 different extracts; each extract was applied at a concentration of 200 µg/ml and incubated under 5% CO_2 at 37ºC for 48 h. DMSO was used as a negative control and suramine was used as a positive control with final concentration of 200 µg /ml for both. All tested samples were performed in three replicates. After 48 h incubation, cell viability was determined by adding (Sigma) tetrazolium salt as cytotoxicity indicator and the absorbance was read at 590 nm with a scanning multi-well spectrophotometer (Thermolab system 354, Finland). Values are expressed as the percentage of mean cell

viability is relative to the untreated cultures. The experiment was repeated using breast cancer cell MCF7, and each experiment was repeated three times for confirmation and validation.

3. Results & Discussions

In this study, the effects of temperature, pressure, SC-CO_2 flow rate on the extraction yield of nutmeg oil were investigated. A recent study by Nik Norulaini [7] showed that extraction parameters have great effect on oil yield and its quality. Thus the selection of the operation parameters was done based on previous study from Machmudaha [8] and on the preliminary experiments that were done in our laboratory (data not shown). The matrix particle size of the samples was fixed to ≤ 1mm, as the reduction of particle size can result in increasing the extraction yield and the extracted compound, and this was revealed by many researchers [9,10]. It was shown from the previous studies that the reduction in the particle size will increase the surface area and may weaken the cell walls and increase the percentage of broken cells, and will lead to the reduction in the mass transfer resistance. Thus the extracted oil will be more accessible to the solvent, as a result, increasing the extraction yield. Also the tested flow rate was in the range of ≤ 1 ml/min, as it was found that varying the flow rate has no significant effect on the extraction yield. Thus, the only parameters that were manipulated were the temperature and pressure and the resulting supercritical extracted oil of the nutmeg were eight extracts. The colour for these extracts were white based on the extraction condition, and all the extracts were in solid paste form. In this study, temperature was selected between 40-50ºC to protect thermolabile compounds and avoid it from degradation. The result of this study reveals a significant difference between the yields obtained at temperatures 50°C and 40°C and at different pressure.

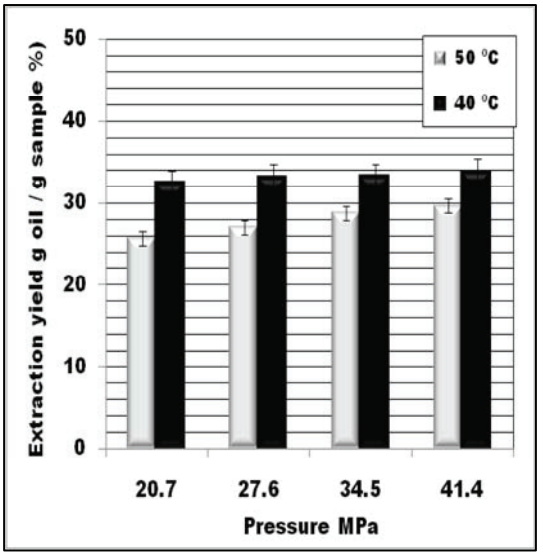

Fig.1. Effect of operating temperature on the extraction yield at different operating pressure using CO_2 flow rate of 1ml/min, with particle size ≤ 1 µm for 60 min

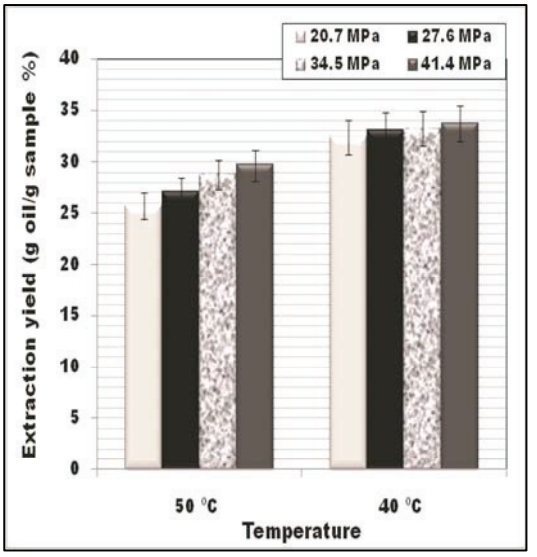

Fig.2. Effect of operating pressure on the extraction yield at temperature 40 and 50 °C using CO_2 flow rate of 1ml/min, with particle size ≤ 1 µm for 60 min

The extraction yield increased with decreasing temperature at constant operating pressure, as shown in Fig. 1. At temperature 40°C, more yields were obtained using different range of pressures, and the highest oil yield was recorded 33.7 g oil /100g of sample at the pressure of 41.4 MPa. While at 50ºC, the oil yield

was less compared to the extracted yield at 40ºC, as the highest oil yield was 29.7 g oil / 100g sample at pressure 41.4 MPa. This might be due to the volatility of some aromatic compounds present in the nutmeg oil. The reason behind this is that when the temperature increased the density of SC-CO_2 will be reduced at a fixed pressure and resulted in increasing volatility. On the other hand, temperature increment will increase the vapor pressure of the compounds to be extracted. Therefore, the tendency of these compounds to move to the fluid phase increased and eventually some of these compounds will vaporize. However, the most important extraction parameter is the pressure that can be used to adjust the selectivity of the SCF. In this study, the operating pressure was selected between 20.7 and 41.4 MPa. The results show that there is no significant difference on the extraction yield among all operating pressures at 40°C, yet there is a significant difference among all pressures at temperature of 50°C as shown in Fig. 2. It is clear that the oil yield increased with increasing pressure at the same applied temperature. The principle rule is, the higher the applied pressure, the greater is the solvent power and the smaller is the extraction selectivity, and when the the pressure is lowered, the selectivity will be higher. This is due to the solubility increment of the extracted oil in the solvent with increasing pressure at high temperature of 50ºC. This result is similar to previous study by Machmudaha [8].

The rat aorta ring assay was employed to study the anti angiogenic properties of the nutmeg oil on the blood vessel formation. The results show that all extracted samples at temperature 40 and 50°C, under different pressure using the particle size ≤ 1 mm has a potent anti angiogenic properties at the concentration of 200 µg/ml as shown in Fig. 3. It was found that at this concentration of the supercritical nutmeg oil the endothelial formation of blood vessels of the rat aorta was significantly inhibited compared to the controls as shown in Fig. 2. The activity of the extracted oil could be related to the high quality of the extraction oil that can be extracted and the extraction condition. The extraction condition at low temperatures maintains the thermolabile active compounds and prevents it from volatilizing at higher temperature resulting in increasing it concentration in the extracted sample. Another factor behind this activity is the antioxidant properties of the nutmeg, as Tomaino [11] had reported the radical-scavenging and antioxidant properties in the DPPH radical assay for the oil of nutmeg. It has been shown that antioxidant considered as a natural angiogenesis inhibitors. In addition, the presence of some component of the nutmeg essential oil like flavonoids, alkaloids [9] that can be extracted only at low temperature between 40 to 50ºC.

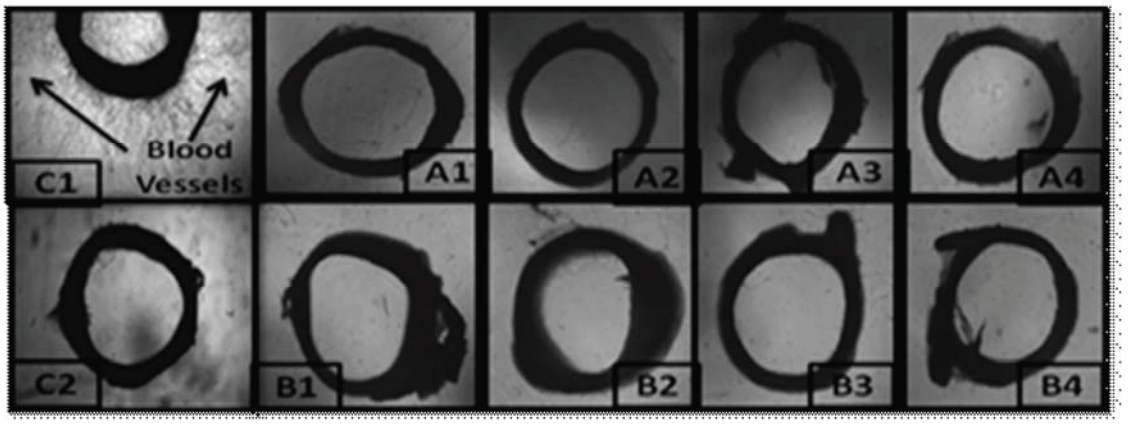

Fig.3. Images of the angiogenesis inhibition of the supercritical extracted nutmeg oil. C1; negative control, a DMSO treated ring shows a full growth of blood vessel. C2: positive control, Suramine treated ring showed 100% inhibition. A1, A2, A3, A4, B1, B2,B3 and B4 are Nutmeg oil samples show inhibition of blood vessel outgrowth from rat aorta

Cell proliferation (cytotoxicity) assay were performed to test the possible cytotoxicity of the supercritical extracted nutmeg oil. A screening for two cancer cells lines was done, these cells were colon cancer cell HCT116 and Breast Cancer cell MCF7. The cells were grown up to 5 d in the presence of 200 µg/ml of each of the four supercritical extract. None of the tested cancer cell was inhibited by the treatment of 200 µg/ml concentration of oil. The principle of the MTT assay using the tetrazolium salts is that the salt are cleaved to formazan dye by cellular enzymes (only in the viable cells) and the level of absorbance directly correlates to the metabolically active cells. This result indicates that all the supercritical extract of nutmeg exhibited no cytotoxic effect on all these cancer cell line and that the nutmeg oil is noncytotoxic and safe for medical use. However, this result supports that by adjusting the extraction condition as in supercritical extraction, a safe and clean extract which is solvent-free with high purity and noncytotoxic can be obtained. The role of a noncytotoxic property of the treatment with anti-angiogenic properties can be a promising treatment for tumor. As the anti-angiogenic agent would help in blocking these vessels, and could suppress tumor growth by preventing the cells from obtaining the nutrients, which is very essential for its growth. On the other hand, the noncytotoxic properties will not have any undesirable side effect on other healthy cells.

4. Conclusion

The extraction yield of nutmeg oil increase with decreasing temperature, but increase with increasing pressure using high temperature using the sample size of ≤ 1 mm. The results of this experiment indicate that supercritical nutmeg oil significantly inhibits the blood vessel formation of the rat aorta and the oil was significantly noncytotoxic, which indicates that nutmeg oil has potential use in the treatment to minimize tumor angiogenesis. Moreover, these results confirmed that supercritical fluid extraction technology can be applied in pharmacology and drug discovery to produce a high quality extracts for the treatment of cancer and many other diseases.

Acknowledgements

The author also would like to acknowledge with thanks the Research Grant (Graduate fund) provided by IPS, Universiti Sains Malaysia, Penang, Malaysia. The author also would like to acknowledge with thanks Shirin and Ziad for the guide and assistant provided**.**

References

[1] Morita T, Jinno K., Kawagishi H, Arimoto Y, Suganuma H, Inakuma T, Sugiyama K. Hepatoprotective effect of myristicin from nutmeg (Myristica fragrans) on lipopolysaccharide/ D-galactosamine-induced liver injury. *J Agric. Food Chem* 2003;**12**:1560-1565.

[2] Bradley D. Angiogenesis inhibitor enters clinical trials. *Journal of Pharmaceutical Science & Technology Today* 2000;**3**: 403.

[3] Edris A E. Pharmaceutical and Therapeutic Potentials of Essential Oils and Their Individual Volatile Constituents: A Review. *Phytotherapy Research* 2007; **21**: 308–323.

[4] Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nature Reviews Cancer* 2008; **8**: 592–603.

[5] Nik Norulaini N A, Md Zaidul I S, Anuar O, Omar A K Mohd. Supercritical enhancement for separation of lauric acid and oleic acid in palm kernel oil (PKO). *Separation and Purification Technology* 2004;**35**: 55–60.

[6]Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation andcytotoxicity assays. *Journal Immunol. Methods* 1983; **65**: 55–63.

[7] Nik Norulaini N A, Setianto W B, Zaidul I S M, Nawi A H, Azizi C Y M, Omar A K M. Effects of supercritical carbon dioxide extraction parameters on virgin coconut oil yield and medium-chain triglyceride content. *J Food Chemistry* 2009;**116**: 193-197.

[8] Machmudaha S, Sulaswatty A, Sasaki M, Goto M, Hirose T. Supercritical CO2 extraction of nutmeg oil: Experiments and modeling. *Journal of Supercritical Fluids* 2006 ; **39**: 30–39.

[9] Spricigo CB, Pinto LT, Bolzan A, Novais A F. Extraction of essential oil and lipids from nutmeg by liquid carbon dioxide. *Journal of Supercrit Fluids* 1999; **15**: 253–259.

[10] Boutin O, Badens E. Extraction from oleaginous seeds using supercritical CO2: Experimental design and products quality. *J. Food Eng* 2009; **92**: 396.

[11] Tomaino A, Cimino F, Zimbalatti V, Venuti V, Sulfaro V, De Pasquale A, Saija A. Influence of heating on antioxidant activity and the chemical composition of some spice essential oils. *J Food Chemistry* 2005;**89**: 549-554

Presented at ICEF11 (May 22-26, 2011 – Athens, Greece) as paper EPF700.