# Sesame seed lignan transformation and recovery under supercritical carbon dioxide

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## Abstract

# Sesame seed lignan transformation and recovery under supercritical carbon dioxide

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Sesame seed is one of the well-known oilseed with rich source of nutritive values. It is ranked 2nd next to flax seed, in its lignan content. It is considered to be one of the most valuable oilseed primarily because of the presence of highly bioactive lignan components. These components gave sesame oil excellent stability due to the strong antioxidant properties. Sesamin and sesamolin are the main lignans in sesame oil, and relatively very small amount of sesamol also exists. Actually, sesamol does not exist naturally in sesame seed, rather it's derived from sesamolin during sesame seed pre-treatment such as roasting, bleaching, and degumming. Even after the treatments only small amount of sesamol is detected in the oil. However small the amount of sesamol is, it exhibits the strongest antioxidant property compared to both sesamin and sesamolin.

For this reason, the acidic conversion of sesamolin to sesamol under supercritical carbon dioxide was performed using solid catalyst (Amberlyst-15) having strongly acidic functional group. Previously, the transformation of sesamolin to sesamol and

samin was performed in toluene, with the addition of strong oxidizing agents such as hydrogen peroxide. However, in this research work, sesamolin under supercritical $CO_2$ was efficiently transformed mainly to sesamol and small amount of other lignans such as samin and sesaminol. Here, no oxidizing agent was used, only small fraction of water was added to furnish hydroxide ion in the solution. The two main advantages of using supercritical $CO_2$ is; first it replaces relatively toxic chemical (toluene) with a green solvent ($CO_2$). Second, product purification is not important, as $CO_2$ can easily be separated by depressurization.

The other important work in this research is, the recovery of water soluble bioactive components from the solid residue (defatted sesame meal) using $CO_2$ assisted hydrothermal process. Defatted sesame meal (DSM) just like sesame oil, it is also rich source of pharmaceutical components. With the ever-growing demand for novel natural drugs, the utilization of green and efficient techniques to obtain highly biologically active natural products from unexplored herbal/medicinal plants is becoming increasingly important. Herein, the efficiency of green hydrothermal extraction (HT-extraction) for the recovery of bioactive compounds from DSM was investigated. The effects of the extraction temperature, extraction time, and ratio of $CO_2$ added on the product yields and physicochemical, and structural properties of the DSM extracts were systematically investigated. In addition, the antioxidant activities of the HT extracts were examined using different *in vitro* methods, such as $DPPH^{\bullet}$ scavenging capacity assay, and $ABTS^{\bullet+}$ scavenging capacity assay, and the correlations between the composition of the HT extract and the antioxidant activities of the resulting extracts were analyzed to determine the origin of the antioxidant

activity of the extracts. Both extraction temperature and time were found to influence the extraction yields and product quality.

The addition of $CO_2$ even at lower temperature has showed the presence of more bioactive compounds and better antioxidant activities were achieved compared to conventional extraction. In vitro, anticancer cell effects on human breast cancer cells were checked for the extract solutions and a promising anticancer effect with lesser effect on normal cells was observed.

**Keyword:** Sesame lignan, antioxidant, bioactivity, transformation, Supercritical carbon dioxide, hydrothermal process

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# Chapter 1. Introduction

## 1.1. Study Background

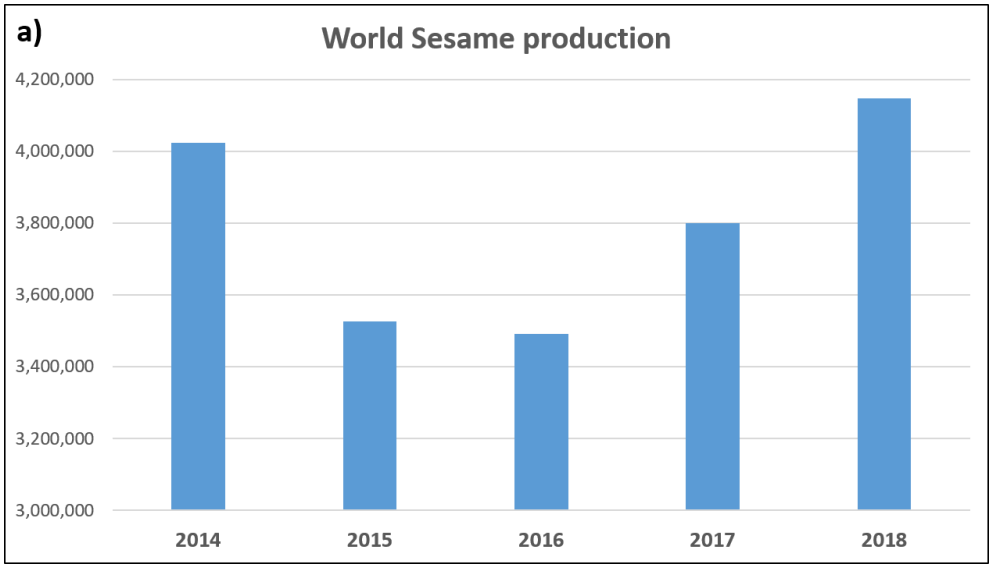
Sesame seed (*Sesamum indicum L.*) is a well-known oilseed with a good nutritive value. It is generally labelled as "queen of oilseeds" mainly due to its high polyunsaturated fatty acid contents, highly nutritious minerals, antioxidant components, delicious nutty aroma, and flavour[1]. It is considered to be one of the most valuable oilseed primarily because of the presence of highly antioxidant components. These components gave sesame oil excellent stability due to the strong antioxidant properties. It is also known that sesame oil has much longer shelf life as compared to other edible oilseeds mainly because of the strong resistance to oxidative rancidity that might occur from contaminations[2,3]. Sesame seeds contains a substantial amount of oil, accounting nearly 50% by weight, protein 25%, carbohydrate 16% and 5% fibrous material and close to 4% moisture. Sesame seed protein content is slightly low in lysine but rich in other amino acids specially methionine, cysteine, arginine and leucine[4]. Fatty acid composition of sesame oil is, linoleic acid (37-47%), oleic acid (35-43%), palmitic (9-11%) and stearic acid (5-10%) with trace amount of linolenic acid[3]. Small amounts of essential minerals, and a high amount of methionine and tryptophan, fibres as well as secondary metabolites such as lignans, flavonoids, and phenolic compounds are also present in sesame seed. Moreover, the seeds are good source of calcium, phosphorus, and iron and are rich in vitamin B, E, and a small amount of trace elements[4]. Sesame oil has a pleasant, mild taste and is highly stable. It has a high content of polyunsaturated fatty acids, oleic, and linoleic acid. Sesame seed is used for a wide collection of

edible products in raw or roasted form and also industrially it is used as input in different sectors such as soaps, lubricants, lamp oil, an ingredient in cosmetics; pharmaceutical uses, and animal feed (the defatted residue). It is also categorized as a healthy food in Asian countries. As a result, it is frequently served, almost daily in small amount along with other main Asian cuisines, such as in Korean foods. It is also commonly consumed in other parts of the world, like the Middle east, Europe and Africa. However, the major sesame seed production is accounted by Asian and African countries. Nowadays, the consumers mostly prefer the high nutritive value products. Consequently, the demand of sesame seeds is higher since it has several nutritional values. Sesame seed is well accepted throughout the world as a natural source of healing ingredients and these ingredients also known as nutraceutical components have been processed industrially and packed in a starch capsules so that they can be easily consumed as food supplements especially for people having weak body defence against chronic diseases[5].

Clinical studies and preliminary reports advocate that sesame seed extract offers a number of potential health benefits and indeed can help one maintain a healthy and sound body balance if regularly and appropriately consumed[6]. Sesame oil is also a source of polyunsaturated fatty acids including omega-3, omega-6 and omega-9[7]. Polyunsaturated fatty acids are necessary for growth and development and strong evidence supports their role in the prevention and treatment of chronic diseases such as coronary heart disease, hypertension, diabetes and arthritis[6]. Sesame oil has been found to take part in lowering blood pressure, reduce cholesterol level, and help maintain normal blood pressure levels. These effects have been primarily attributed to the naturally high polyunsaturated fat content found in sesame oil. Vitamin E,

which is major constituents of sesame oil nutraceutical components, may also reduce cancer risk and preliminary evidence supports the role of vitamin E in the prevention of Alzheimer's disease and cataracts[8].

World sesame seed consumption is gradually increasing mainly due to the lifestyle changes among consumers and sound scientific evidence for the beneficial health effects of the seed have created a change in the people's consumption patterns and increasing health awareness. Due to the increased demand of sesame seed throughout the world, in recent times its production has been drastically increased. As a result, sesame oil has been ranked the top 10 of important oilseed in the world's oil market. The world sesame production is about 5,532,000 metric tons (MT) behind soybean, groundnut, cottonseed, sunflower, linseed, and rapeseed, in the quantity of world oilseed production[5]. The average sesame productivity of the world's top producing countries within the last five years (2014 to 2018) is given in figure1.1.



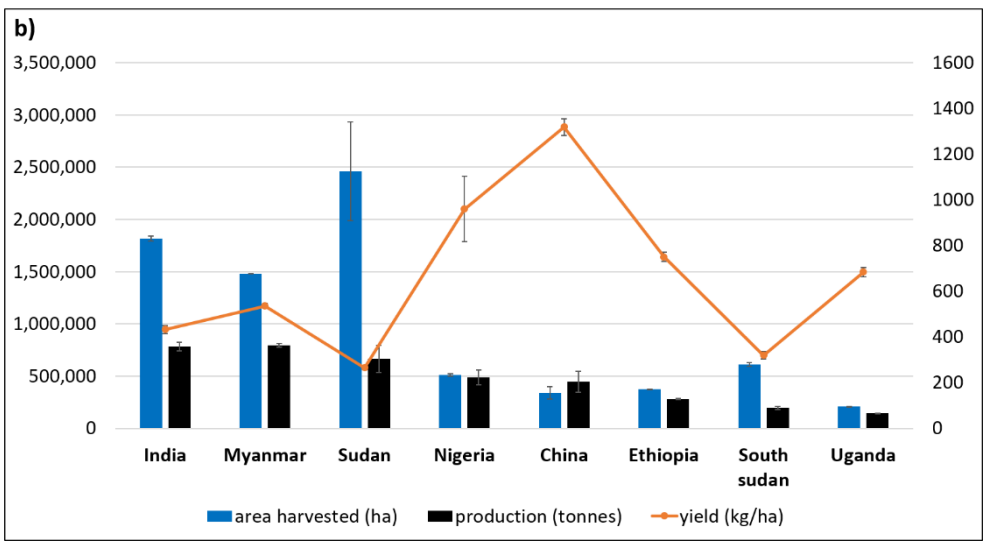




Figure 1- 1 Trend of sesame production values in top producing countries during the last five years (Average 2014–2018) Source: Food and Agriculture Organization Statistical Databases (FAOSTAT), 2020 [9]

Generally, the global herbal medicine markets are continually growing because of their organic nature (not synthesised) and the reliable scientific evidences for its health effects. For example, recent clinical and epidemiological studies showed that plant-based source of fruits and vegetables are rich reservoir of phytochemicals, which are major bioactive compounds, and proved that their consumption helps prevent development of age-related diseases and reduce the risk of chronic diseases. In this regard, the pharmaceutical and biotechnology industries have processed a wide array of biologically active components from different plant sources. These bioactive compounds are often produced using traditional extraction techniques of solid-liquid extraction, maceration, and soxhlet extraction using organic or aqueous solvents. However, the traditional techniques often require long extraction period and usually use a large amount of toxic solvents (*e.g.*, methylene chloride, hexane, and chloroform), of which the latter are often costly, in addition it causes environmental pollution and health problems. The traditionally processed extracts require subsequent concentration and clean-up prior to analysis and use. Moreover, bioactive compounds are typically present at low concentrations, and recovery yields of the bioactive compounds using traditional extraction methods are not satisfactory. This is because bioactive compounds are typically highly sensitive and thermolabile, thus the long extraction period under harsh conditions often cause severe degradations. In addition, the narrow solvation power of a given solvent limits the extraction of wide range of bioactive compounds. Therefore, to comply with the principles of green chemistry, there is an urgent need for the development of alternative sustainable, non-toxic, environmental-friendly, and highly-efficient extraction techniques for the recovery of bioactive compounds from herbal plants.

Among several alternative extraction techniques, hydrothermal extraction (HT-extraction) is a highly promising and green technique in technical and economical perspectives; the use of non-toxic water, short extraction time, and the possibility of using small amount of solvent associated with hydrothermal process as compared to traditional extraction techniques make it high-throughput and green process with no concern over hazardous waste disposal problem. In addition, its tunable physicochemical properties, high selectivity, and simplicity which provide advantages over traditional and modern extraction techniques. In hydrothermal process, the polarity (dielectric constant) of water decreases with increasing in temperature and consequently leads to an increase in solubility of a wide range of the analytes such as polar, moderately polar and non-polar organic compounds. In addition, with increasing temperature, surface tension and viscosity of the solvent decreased significantly, while the self-diffusivity of water increased; this allows enhanced penetration into the solid matrix, which can enhance extraction of target compounds. Therefore, HT-extraction has demonstrated the ability to selectively extract different types of organic compounds by manipulating process parameters. On the other hand, at high temperatures, where extraction yields often increase, the risk of degradation of some bioactive compounds also increases at an extended period of extraction time. In this sense, the extraction temperature and time are critical factors that affect the high efficiency and selectivity in the HT-extraction. Moreover, extraction efficiency depends on the nature of the sample material used or extent of degradability for the extract components. The target components in sesame seed are known as lignans. These components are present in sesame seed

both in polar and nonpolar forms. Hence, the importance of defatted sesame residue cannot be neglected.

## 1.2. Sesame seed lignans

Lignans (shown in Figure 1-2) are chemically described as a group of phenyl dimers in which C6–C3 units are linked by the central carbon of their propyl side chains [1]. Sesame lignans are the second most abundant naturally occurring plant sourced antioxidants next to flax seeds[10]. They are derived biosynthetically from phenyl-propanoids. Most of these lignans occur freely in the oil part, while the remaining significant portion of them co-exist with sugars to form glycosides. The glycosylated lignans are residing in the defatted residue, which is often considered as a by-products of the oil production.

Most of the lignans in sesame seed are categorized as fat soluble aglycons and they elute into the oil during extraction. However, the remaining are glycosylated lignans, and are isolated from the sesame residue (oil free meal). The major aglycon lignans are sesamin and sesamolin while minor lignans such as sesamol, samin, sesaminol, sesamolinol, pinoresinol, and episesamin are almost entirely derived from the major lignans during sesame seed roasting and oil refining processes. Whereas the main lignan glycosides, mainly localized in the defatted sesame meal, include monoglucoside, diglucosides, and triglucosides of sesaminol. Sesaminol triglucoside, and sesaminol diglucoside are the most abundant lignan glycosides in sesame. Sesame seed also contain biologically active tocopherol, mainly γ-tocopherol. Tocopherols (also referred as vitamin E) are very much known for their antioxidative properties in our body. The composition of sesame seed functional components is given in Table 1.

Table 1 Functional components in sesame seed and sesame oil

| Bioactive components | Name of component | Quantity | | Reference |
|---|---|---|---|---|
| | | Sesame seed (mg $g^{-1}$ seed) | Sesame oil (mg $g^{-1}$) | |
| Lignan | Sesamin | 5.71 – 7.12 | 6.20 | [11,12] |
| | Sesamolin | 2.97 – 3.98 | 2.45 | |
| | Sesamol | 1.20 – 1.87 | - | |
| | Sesaminol | 1.40 – 2.22 | 0.01 | |
| | Sesaminol monoglucoside | 0.085 - 0.23 | - | [13,14] |
| | Sesaminol diglucoside | 3.23 – 9.57 | - | |
| | Sesaminol triglucoside | 0.11 – 4.93 | - | |
| Tocopherol | α-tocopherol | - | - | [15] |
| | β-tocopherol | - | - | |
| | γ-tocopherol | 521 – 983 µg.$g^{-1}$ | 680 µg.$g^{-1}$ | |
| | δ-tocopherol | - | - | |
| Polyunsaturated fatty acids | Palmitic acid (16:1) | 9.4% | 14.45% | [11] |
| | Oleic acid (18:1) | 39.1% | 50.54% | |
| | Linoleic acid (18:2) | 40% | 45.50% | |
| | Linolenic acid (18:3) | 0.46% | 0.85% | |
| Phytosterols | β-sitosterol | 2.63 | 3.35 | [16] |
| | Campesterol | 0.7 | 1.00 | |
| | Stigmasterol | 0.28 | 0.37 | |
| | Sitostanol | - | - | |
| | Campestanol | - | - | |
| Total phytosterols | | 4.72 | 5.33 | |
| Minerals | Ca | 4.21 | | [11] |
| | Fe | 0.06 | - | |
| | Zn | 0.03 | - | |
| | P | 4.45 | - | |
| | K | 3.85 | - | |
| | Na | 0.08 | - | |
| | Mg | 2.21 | - | |
| | Cu | 0.41 | - | |
| | Mn | 0.02 | - | |

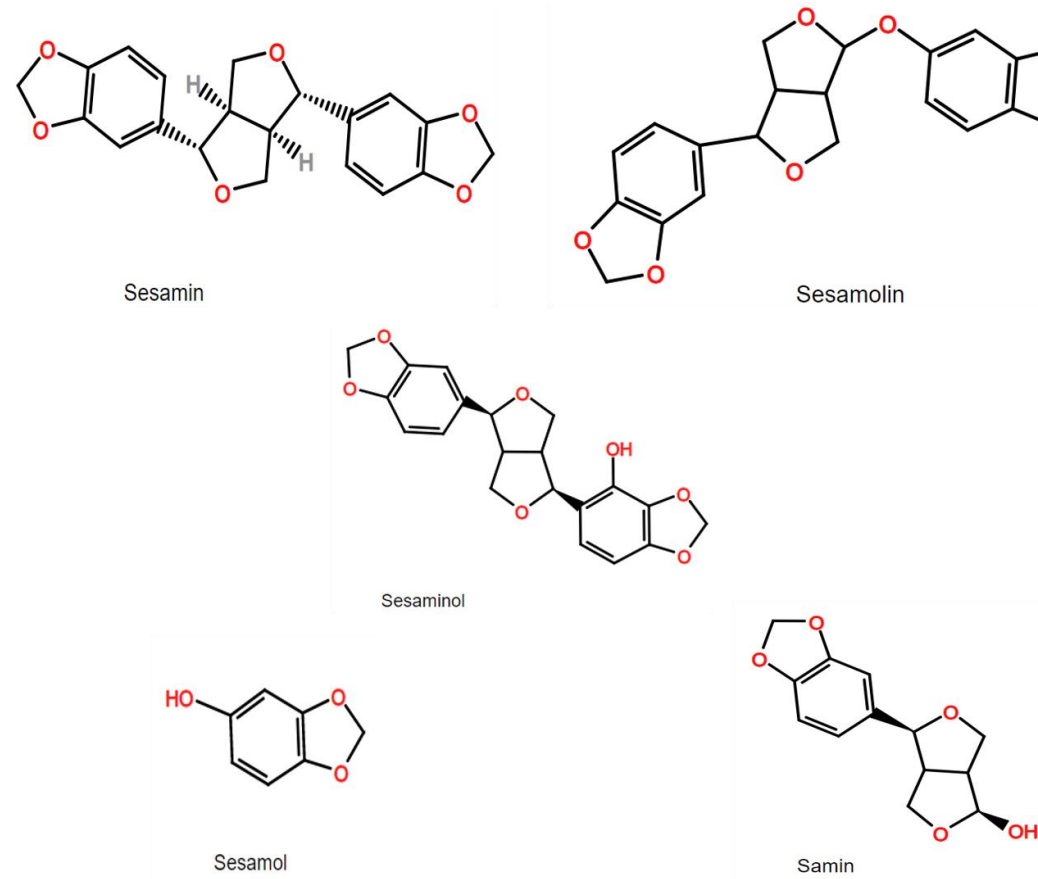



Figure 1- 2 Chemical structure of major sesame oil lignans and their derivatives

### 1.2.1. Sesame oil lignan recovery

Due to the high pharmacological importance of sesame seed lignans, especially sesamin and sesamolin, there have been numerous works regarding the isolation and purification of these lignans from sesame seeds, sesame cake (defatted meal), and sesame oil[17,18]. Sesame seed is rich source of strong bioactive compounds and antioxidants. Antioxidants are widely used food additives to improve food stability and extend the shelf-life of oxygen-sensitive foods[19]. Generally, antioxidants, both synthetic and natural, are very important chemical compounds to prevent oxidative degradation and consequently extend the shelf-life of foods.

The extraction of quality bioactive product from plant sources rely on the selection of suitable solvent type, proper extraction technique and optimized extraction conditions. The need for highly nutritional, having good pharmaceutical value products is on the rise. Especially, in the highly developed countries it is becoming customary to regularly get essential nutrients as a form of capsuled food supplements. Hence, lignans from different oilseeds, such as flaxseed, sesame seed, and sunflower seeds have been investigated for their rich lignan sources[20,21]. However, the major problem with regard to the recovery of oilseed lignans, is that the lignan content in most seeds is very low, which means a highly efficient mechanism is required to adequately recover the components. The other reason is due to the complexity of plant inner structure, it's often not easy to exhaustively extract the lignans from the inner solid matrix of the seed. Therefore, a series of pre-treatment techniques could be employed before components get recovered.

During sesame oil production the non-polar lignan components are eluted along with the oil. Though, the lignans are eluted with the oil, their constituent is very small,

usually less than 1% by weight of the total sesame oil[22]. Therefore, due to the very small amount of lignan presence in sesame oil, a highly effective method of lignan recovery should be employed. For this reason, special attention is given to the type of solvent used, its solvation power, as well as the recovery technique and operating conditions.

#### 1.2.1.1 Conventional lignan recovery techniques

It is the most widely used technique in the recovery of polyphenolic components mainly from plant sources. Steam distillation and soxhlet extraction using organic solvents have been commonly used in the recovery bioactive aglycon phenolic compounds[23]. However, both steam distillation and organic solvents are not the best choices due to process inefficiency, and environmentally unfriendly solvent usages respectively. However, soxhlet extraction still offers certain advantages over other techniques such as, a constant flow of fresh solvent to the sample can be easily maintained, improving the continuous contact of solvent with sample at elevated temperature that favours the extraction of phenolic compounds; (2) maintaining a constant and uniform heat effect on the sample; (3) the final extract can be easily separated from the residue, hence does not require filtration; and (4) several samples can be treated in parallel using relatively low cost and easy operational processes compared to other extraction techniques. However, this technique has some disadvantages as the extraction requires long extraction time and it uses large amounts of solvents at boiling point, and this makes it costly. Furthermore, the high temperature could sometimes degrade the extracted product if they are sensible to thermal effects. Additionally, the process is not convenient to perform agitation hence it’s extremely time-consuming and inefficient technique. Finally, the organic

solvents used in the extraction will need to be removed from the final product by evaporation, posing both economic and environmental burden. Especially, due to the health risk associated with the presence of even minute residue solvents in the final product, the process requires utmost caution and check-ups, and that definitely incurs addition cost.

Hence, the appropriate solvent type should be selected to effectively and selectively recover the lignan components from the plant source[24,25]. For this reason, the solubility parameter can be used to scientifically select the best solvent. The solubility parameter of some organic solvents and solubility parameter of major sesame oil lignans as well is presented in Table 3. The solubility parameters for the lignans was calculated based on the group contribution method[26]. The component solubility parameter for selected functional groups and elements is listed in Table 2. After estimating the solubility parameter of a component (lignan), the best solvent can be chosen by matching the solubility with the solvent solubility parameter.

The Hoftyzer and Van-Krevelen method can be used to predict the three solubility parameter components through the group contribution method. Equations 1 to 3 were used to determine the solubility parameter components.

$$\delta_d = \frac{\sum F_{di}}{V} \quad (1)$$

$$\delta_p = \frac{\sqrt{\sum {F_{pi}}^2}}{V} \quad (2)$$

$$\delta_d = \sqrt{\frac{\sum E_{hi}}{V}} \quad (3)$$

Where,

$F_{di}$, is the group contributions to dispersion component of molar attraction constant

$F_{pi,}$ is the group contributions to polar component of molar attraction constant and

$E_{hi}$, is the group contributions to hydrogen bonding energy component and

V, is the molar volume ($cm^3$/mol)

### 1.2.1.2 Non-conventional lignan recovery techniques

Different extraction methods have been used to effectively recover the bioactive compounds from plant sources[27,28]. The quantity as well as quality of the bioactive compounds can be highly affected by the type of solvent used and the extraction conditions employed. Non-conventional techniques are alternative extraction methods that can efficiently recover the bioactive compounds and overcome the challenges from using conventional techniques. Among the non-conventional techniques, pressurized liquid extraction (PLE) and supercritical fluids extraction (SFE) are among the most recently used and efficient techniques.

Pressurized liquid extraction

Pressurized liquid extraction is also known as subcritical water extraction. It's an extraction technique which uses pure water as an extraction solvent, at temperatures above the boiling point but below the critical point of water and at a pressure high enough to maintain the liquid state[29].

The dielectric constant of water is significantly decreased under pressurized conditions, and its polarity reaches close enough to the values like those of organic solvents.

Table 2 Solubility parameter according to the group contribution method[30].

| Group | $F_d$ ($J^{1/2}$. $cm^{3/2}$). $mol^{-1}$ | $F_p$ ( $J^{1/2}$. $cm^{3/2}$. $mol^{-1}$) | $E_h$ (J/mol) |
|---|---|---|---|
| -CH2- | 270 | 0 | 0 |
| >CH- | 80 | 0 | 0 |
| =CH | 200 | 0 | 0 |
| =C< | 70 | 0 | 0 |
| -OH | 210 | 500 | 20000 |
| -O- | 100 | 400 | 3000 |
| CH3 | 420 | 0 | 0 |
| -COOH | 530 | 420 | 10000 |
| -N< | 20 | 800 | 5000 |
| >C=O | 290 | 770 | 2000 |
| Phenyl | 1270 | 110 | 0 |
| C | -70 | 0 | 0 |
| -F | 220 | 0 | 0 |
| -Cl | 450 | 550 | 400 |
| -Br | 550 | 0 | 0 |
| -CN | 430 | 110 | 2500 |
| -OH | 210 | 500 | 20000 |
| -O- | 100 | 400 | 3000 |
| -COH | 470 | 400 | 4500 |
| -CO- | 290 | 700 | 2000 |
| -COO- | 390 | 490 | 7000 |
| HCOO- | 530 | 0 | 0 |
| -NH2 | 280 | 0 | 8400 |
| -NH- | 160 | 210 | 3100 |
| -N< | 20 | 800 | 5000 |
| -NO2 | 500 | 1070 | 1500 |
| -S- | 440 | 0 | 0 |
| >PO4- | 740 | 1890 | 13000 |
| Ring (5) | 190 | 0 | 0 |
| Ring (6) | 250 | 0 | 0 |
| Benzene | 1270 | 110 | 0 |

Table 3 Solubility parameters of sesame oil lignans and typical organic solvents

| **Lignans** | **$\delta_d$ ($MPa^{1/2}$)** | **$\delta_p$ ($MPa^{1/2}$)** | **$\delta_h$ ($MPa^{1/2}$)** | **$\delta_t$ ($MPa^{1/2}$)** |
|---|---|---|---|---|
| Sesamol | 20.85 | 8.42 | 17.03 | 28.2 |
| Sesamin | 20.95 | 4.31 | 8.9 | 23.17 |
| Sesamolin | 21.04 | 4.58 | 9.53 | 23.55 |
| **Solvents** [25] | | | | |
| Methanol | 15.10 | 12.30 | 22.30 | 29.60 |
| Acetonitrile | 15.3 | 18.0 | 6.1 | 24.3 |
| Ethanol | 15.8 | 8.8 | 19.4 | 26.5 |
| Acetone | 15.5 | 10.4 | 7.0 | 20.0 |
| DMSO | 18.4 | 16.4 | 10.2 | 26.7 |
| Water | 15.6 | 16.0 | 42.3 | 47.8 |
| Ethylene glycol | 17.0 | 11.0 | 26.0 | 32.9 |
| 1-butanol | 16.0 | 5.7 | 15.8 | 23.1 |

In pressurized water conditions, PLE acquires solvent properties very convenient characteristics for the extraction of phenolic compounds. PLE has many advantages compared to conventional extraction methods, such as high extraction efficiency, higher yield, and use of green solvent.

## Supercritical carbon dioxide extraction

Supercritical fluid extraction (SFE) eliminates the disadvantages of conventional solvent extraction, which could contribute to the degradation of heat sensitive compounds and might also leave traces of toxic solvents in the final product[31]. This is a concern for food and medicinal extracts. However, using supercritical carbon dioxide and by just changing the operating pressure and/or temperature, the low solubility of oil can be increased and also the extraction rates can be greatly improved. Therefore, the functional and nutritional components of sesame oil can be easily recovered along with the extracted oil. Previous researches have reported on the extraction of oils from different seeds with supercritical $CO_2$[32]. The most important advantages of carbon dioxide are, its non-flammability, which makes it a significant safety advantage in using it as a solvent. The supercritical carbon dioxide extraction process (figure 1-3) is briefly discussed as follow. Liquefied $CO_2$ in the cylinder is first cooled by a circulator to keep $CO_2$ in liquid state before pumping it to the extractor. Just before introducing $CO_2$ to the extraction cell, it's preheated so that it attains a supercritical condition. After the extraction time ends, $CO_2$ is slowly discharged (using a back pressure regulator) from the extraction cell to the separator. Here, the product is collected by depressurization of $CO_2$, at which point $CO_2$ is changed to gaseous state.

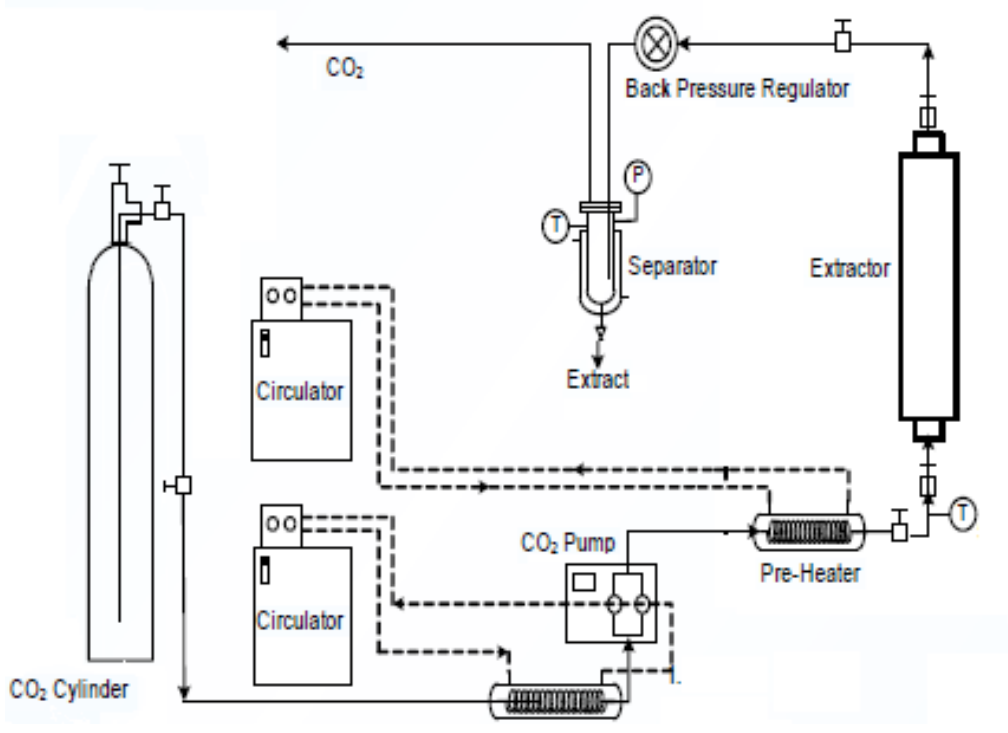



Figure 1- 3 Supercritical carbon dioxide extraction flow diagram

## 1.3. Bioactivity and anticancer applicability

Bioactive compounds are molecules that have high therapeutic potential, while reducing pro-inflammatory state, oxidative stress, and metabolic disorders in our body[33]. “Epidemiological studies indicate that high consumption of foods rich in bioactive compounds with antioxidant activity, including vitamins, phytochemicals, and mainly phenolic compounds, such as flavonoids and carotenoids, has a positive effect on human health and could diminish the risk of numerous diseases, such as cancer, heart disease, stroke, Alzheimer’s, diabetes, cataracts, and age-related functional decadence”[34]

Cancer is one of the most prevalent health problems and also responsible for the significant decrease in the life quality of our society. According to WHO, approximately one-third of total death caused by cancer are related to bad eating habits and lack of frequent physical exercises[35]. Hence by properly consuming nutritious food products and performing regular physical exercises can greatly reduce the risk of cancer. So, generally balanced nutrition plays a fundamental role in reducing cancer and can maintain the well-being of an individual.

Among the vast majority of bioactive compound sources, flaxseed, Allium hookeri, and sesame seeds have been the focus of many researches. These plants have been considered as the potential sources of components for cancer treatments. These and other different research works have been successfully applied, *in vitro* treatment of human cancer cells using extracts from flaxseed, allium hookeri extract, and sesame seed extracts[29,36,37].

## 1.4 Sesame oil lignan transformation

In sesame oil, the main oil soluble lignan components are sesamin and sesamolin (Table 1), which accounts approximately 58% and 38% of the total lignan respectively. Of the remaining 4%, sesamol accounts the major portion. This is because sesamol can only be derived from sesamolin during heat treatment. In fact, sesamol is almost undetected in sesame oil from unroasted seed[38]. Hence, it is derived from sesamolin during sesame seed pre-treatments, such as roasting and/or during oil refining processes like bleaching and degumming. Even though sesamol is present in small amount in sesame oil, it has been reported to show strong antioxidant and free radical scavenging activities among other sesame lignans (Table 5). Therefore, one objective of this research is to transform sesamolin (a less antioxidant) to sesamol (strong antioxidant) under supercritical carbon dioxide condition. So far, thermal treatment in acidic media is the only mechanism reported to transform sesamolin to sesamol.

### 1.4.1. Sesame oil lignan transformation in subcritical water

Here, subcritical water was proposed for lignan transformation for the fact that, enough heat can be supplied to the reaction, water can be used both as reaction media and reactant during the hydrolysis of sesamolin, and the tunability of water under subcritical conditions makes it easier to manipulate its solvation power. The changes in the physical properties of water such as the dielectric values, the density, and diffusivity could facilitate the interaction between the immiscible oil component and the moderately polar subcritical water. Hence, the main parameter is temperature, used to investigate sesamolin transformation in the presence of water.

The use of subcritical water provides a number of advantages over other lignan transformation mechanisms such as short reaction time, higher product quality, cheaper cost of reaction media (water) and an environmentally benign technique. In addition, water at high temperature condition is known to show acidic properties hence producing a favourable condition for the hydrolysis of sesame lignan (sesamolin) without the need for using additional acid. However, there are some disadvantages arising from using subcritical water at such high temperatures. The first is the hydrolysis effect of fatty acid components of sesame oil. The high temperature condition along with acidic pH could produce free fatty acids and glycerol, which in turn could compromise the oil quality. The other disadvantage is degradation of produced lignan (sesamol, and samin) since subcritical water is carried out using hot water in the ranges of boiling point to near the critical points of water, under high pressure (usually from 10 to 60 bar) to maintain water in the liquid state, it has a negative impact on the stability of lignan components that are sensitive to very high temperatures and the exposure to the extreme condition for prolong period results degradations. Furthermore, emulsification is also a serious problem when oil comes in contact with water at high temperature and pressure. The oil is partially emulsified with water, and sesame oil could easily be discharged from the reactor before complete lignan conversion is achieved. Generally, in subcritical water lignan transformation product recovery, separation, and purification are main challenges in addition to the high cost required for the process.

### 1.4.2. Sesame oil lignan transformation in Toluene

It has been shown that sesamolin under heat treatment can be hydrolyzed to sesamol and samin in the presence of water. However, it will convert to sesaminol, instead of sesamol in anhydrous conditions[39]. Therefore, water is important component in the transformation of sesamolin to sesamol. However, due to water-oil immiscibility an ideal reaction media is required to bring to contact the reactants (oil and water). Previous researches used toluene as a reaction media in sesamolin transformation[39,40]. Toluene has good miscibility with water and at the same time it is good solvent for nonpolar materials such as sesame oil. Hence, toluene is used for both hydrous and anhydrous decomposition of sesamolin. As shown in (Figure 1-4), Acidic decomposition of sesamolin, without the use of solid catalyst (instead using sulphuric acid) was performed in the presence of hydrogen peroxide as well as without the addition of hydrogen peroxide. In the acidic condition the specific formation of 7R,7′R-samin is favoured, which is an isomer of 7R,7′S-samin[40]. As reported by H.Y Tsai, et al, the possible role of hydrogen peroxide in the formation of samin in the acidified hydrogen peroxide was also explored by directly reacting sesamolin with 30% sulphuric acid with and without the addition of hydrogen peroxide. It was found out that sesamolin was mainly converted to sesamol and 7R,7′S-samin in which case, it showed that hydrogen peroxide might be related to the epimerization of 7R,7′S-samin under acidic conditions. This suggests that hydrogen peroxide alone was capable of converting sesame lignans.

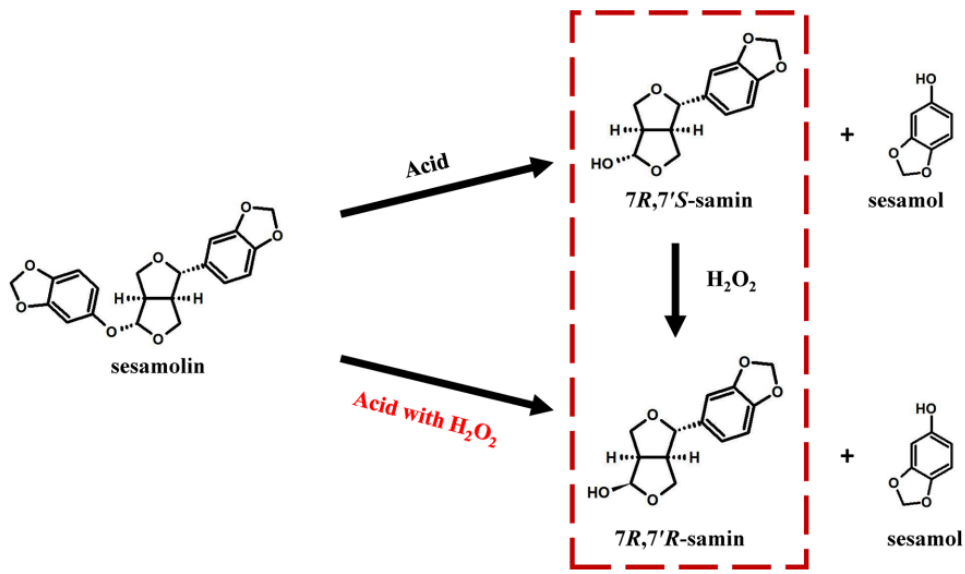



Figure 1- 4 Transformation of sesamolin under general acidic conditions or catalyzed with hydrogen peroxide under acidic conditions[40].

The possible lignan transformation mechanism is depicted in Figure 1-4, which clearly shows the influence of oxidizing agent such as hydrogen peroxide on the selective transformation of lignan. Generally, this reaction favours the production of samin not sesamol. Additionally, to date there is no research finding that shows the potential use of samin.

### 1.4.3. Sesame oil lignan transformation in supercritical $CO_2$

Carbon dioxide in its liquid or supercritical state has been used as a benign reaction medium for different catalytic reactions[41,42]. Supercritical carbon dioxide is a very good solvent for non-polar components such as oil as its solubility characteristics matches with the solubility of non-polar chemicals. The physicochemical properties of supercritical carbon dioxide (Table 4), makes it ideal condition for the reaction of catalytic processes. Additionally, the swelling effect of compressed $CO_2$ on polymeric compounds could facilitate mass transfer of components between the solid catalyst and oil in the supercritical reaction phase.

In industrial processes, in particular product syntheses are often carried out in solution phase, and organic solvents are usually the first choice. But, these solvents are often associated with serious problematic issues mainly related to their toxicity, flammability, and environmental hostility. On the other hand, the solvent can play a very important role in the stabilization of reactive intermediates and often has a decisive influence on the rate and selectivity of a particular reaction.

Therefore, the choice of solvent is generally made in accordance with the effectiveness of the solvent media in bringing the target product with maximum conversion and selectivity, keeping the product stability during the process time and minimizing unwanted side reaction that could reduce the final yield. In addition, nowadays the concern with safeguarding the environment has also been given priority. Hence, the use of "green solvents" such as supercritical carbon dioxide is highly encouraging. In this research work the prior objective was to replace organic solvent (in this case toluene) with a better and nonhazardous solvent, such as supercritical carbon dioxide without compromising the percentage conversion of sesamolin. Secondly, it's aimed to avoid the use of additional oxidizing agents such as hydrogen per oxide or potassium permanganate. But, the problem with regard to using an oxidizing agent is that during lignan transformation the unsaturated fatty acid components of sesame oil can be easily epoxidized[43]. Epoxidation is undesired side reaction that could greatly affect the oil quality, or even hinder lignan conversion. Thirdly, in the case of supercritical carbon dioxide, there is no need for additional product separation. Only centrifugation is need for the catalyst separation and depressurization for carbon dioxide separation. In this regard, supercritical carbon dioxide is envisioned to replace toluene as both reaction media and provision of acidic condition by producing carbonic acid due to the increased solubility of $CO_2$ in water at high pressure[41].

Table 4 Comparison of the physical properties of $CO_2$ at different states [24]

| State | Diffusivity ($cm^2.s^{-1}$) | Viscosity ($g.cm^{-1}.s^{-1}$) | Density ($g.cm^{-3}$) |
|---|---|---|---|
| Liquid | $(0.2 – 2)10^{-5}$ | $(0.2 – 3)10^{-2}$ | 0.6 – 1.6 |
| Supercritical fluid | $7 \times 10^{-4}$ (liquid like) | $3 \times 10^{-4}$ (gas like) | 0.4 – 0.5 (liquid like) |
| Gas | 0.1 – 0.4 | $(1 – 3)10^{-4}$ | $(0.1 – 2)10^{-3}$ |

Table 5 Sesame oil lignan antioxidant capacity comparison [44]

| No. | Test assay | Comparison | References |
|---|---|---|---|
| 1 | DPPH | Sesamol > Sesamin > Sesamolin | [45] |
| 2 | DPPH | Sesamol > Sesamin > Sesamolin >STG >SDG | [46] |
| 3 | Superoxide scavenging | Sesamol > Sesamolin > Sesamin | [38] |
| 4 | TEAC[a] | Sesamol > Sesamolin > Sesamin | [47] |
| 5 | Methyl linoleate oxidation | Sesamol > Sesamin > Sesamolin > α tocopherol | [48] |

[a] TEAC, Trolox equivalent antioxidant capacity assay

STG, sesaminol triglucoside

SDG, sesaminol diglucoside

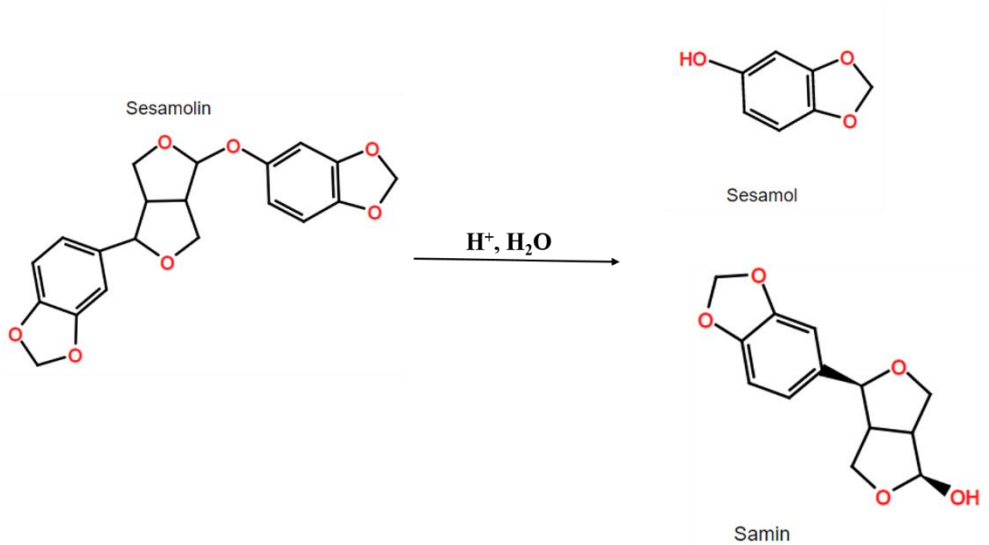



Figure 1- 5 Conversion of sesamolin to sesamol, and samin in acidic media in the presence of water

## 1.5 Research objectives

The general objective of this research is to propose a green alternative solvent for the recovery, transformation and extraction of lignans and bioactive components from sesame seed.

The specific objectives are;

- To recover and quantify sesame seed lignans (oil soluble lignans) using supercritical carbon dioxide.
- To upgrade the oil quality by transforming sesamolin through lignan transformation using hydrolysis of sesamolin under supercritical carbon dioxide to a stronger antioxidant component (sesamol).
- To replace toluene with a green solvent (Carbon dioxide)
- To perform lignan transformation without the use of an oxidizing agent
- To enhance the bioactive components of DSM extract
- To investigate the anticancer effect of water soluble bioactive components obtained from the defatted sesame meal.

# Chapter 2. Experimental

## 2.1. Chemicals and reagents

Sesame oil extracted using supercritical carbon dioxide was used as a raw material. Standard chemicals such as sesamin (≥ 98%, HPLC), sesamolin (≥ 97%, HPLC), sesamol (≥ 98%, HPLC), and Amberlyst 15 ion-exchange resin (strongly acidic, macroreticular resin with sulfonic acid functionality) were purchased from Sigma Aldrich-Korea (Seoul, Korea). Samin (≥ 99%, HPLC) and sesaminol (≥ 99%, HPLC) were purchased from Nagara Science Co., Ltd (Gifu, Japan). HPLC grade water was purchased from J.T. Baker (USA) and others such as HPLC grade methanol and Toluene were all purchased locally from Samchun Pure Chemicals, CO, LTD (Seoul, Korea). Carbon dioxide (99.99%) was purchased from Hyupshin Gas Industry (Seoul, Korea).

## 2.2. Reaction procedure and product collection

Prior to lignan transformation, the effect of extraction temperature and pressure on the composition of sesame oil lignans was analyzed. The extraction, process shown in (Figure 1-3) was performed using a roasted sesame seed powder as a raw material and extracted at temperatures of 40, 50, and 60 $^{o}$C and pressure of 200, 300, and 400 bar for extraction time of 30, 60, and 90 minutes. The lignan composition was analyzed at each time using high performance liquid chromatography technique.

Then, sesame oil lignan transformation was performed in a 23 mL, stainless steel batch reactor. Sesame oil was first put in the reactor followed by Amberlyst-15 catalyst (5% weight of the oil), and then 0.25 g of water is added to it. Two metal beads of 3 mm size were also used to facilitate the mixing of reactants and catalyst component. Then, the caps are tightly closed and then connected to a $CO_2$ pump, in

which about 10g of carbon dioxide is pumped in to the reactor. As shown on Figure 2-1, the reactor is designed in a way, that it can be easily connected with a pressure gauge and a valve (used to introduce and discharge $CO_2$). The reactor is put in a metal rack and immersed in hot oil bath.

The reaction temperature was 80, 100, and 120 $^{o}$C and pressure of 160,180, and 200 bar respectively. The reaction time was 10, 20, 30, 60, 90, and 120 minutes.

### 2.2.1. Product analysis

Sesame oil lignan composition was analyzed using high performance liquid chromatography (HPLC). The extracts were filtered using 0.45 μm syringe filter to get rid of insoluble matters before HPLC analysis. The extract analysis was performed using an Agilent 1200 HPLC with a UV-Vis detector (Agilent Technologies, Inc., USA). Chromatographic separations were carried out on a reverse phase Zorbax Eclipse Plus-C18 column (solvent saver plus: 4.6 mm x 150 mm x 5 μm) (Agilent, USA). The mobile phase gradient was obtained with HPLC grade water (solvent A) and HPLC grade methanol (solvent B). Following the injection of 20μL of sample, solvent B was increased from 45% to 90% over 20 min, then to 100% within 3 min, and then it was isocratic for another 2 min, and finally decreased to 45% within 10 min. A flow rate of 1 mL/min was used, and the lignan components were detected at a wavelength of 290 nm at 30 ℃.

The lignan conversion and yield of sesame oil lignans was calculated as follows:

$$Sesamolin\ conversion\ (mole\ \%) = \frac{moles\ of\ sesamolin\ converted}{initial\ moles\ of sesamolin} * 100 \qquad (4)$$

$$Sesamol\ yield\ (mole\ \%) = \frac{actual\ moles\ of\ sesamol}{theoretical\ moles\ of sesamol} * 100 \qquad (5)$$

$$Samin\ yield\ (mole\ \%) = \frac{actual\ moles\ of\ samin}{theoretical\ moles\ of\ samin} * 100 \qquad (6)$$

$$Sesaminol\ yield\ (mole\ \%) = \frac{actual\ moles\ of\ sesaminol}{theoretical\ moles\ of sesaminol} * 100 \qquad (7)$$

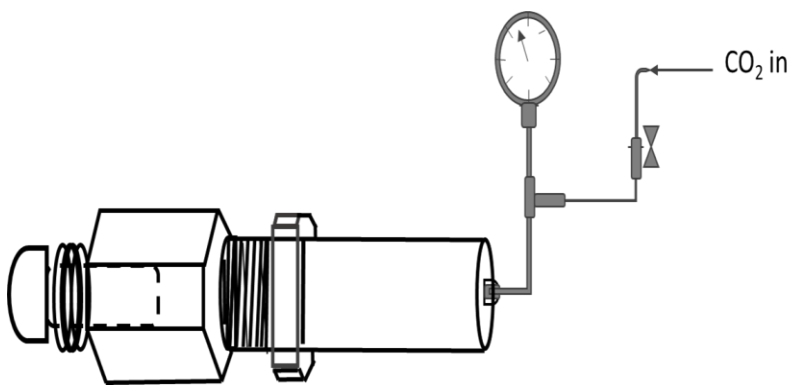



Figure 2- 1 Batch reactor (23mL) connected to pressure gauge and $CO_2$ inlet tube

## 2.3. Result and discussion

### 2.3.1 Supercritical carbon dioxide extraction of sesame lignan

The effects of extraction parameters on sesame lignan composition is presented on Figure 2-2. Generally, the higher lignan content was obtained at the beginning of each extractions, while it showed gradual decrement with increase in reaction time. The highest sesamin content was recorded at 40 $^{o}$C, 400 bar and 30 minutes. However, with increase in extraction time, sesamin content was decreased even at higher pressures. Despite the decrease in sesamin, over all oil yield showed gradual increment with increase in extraction time. Similarly, sesamolin content was higher at the beginning of the processes, except for the lower temperature (40 $^{o}$C). The highest sesamolin was recorded at 50 $^{o}$C, 400 bar and 30 minutes. The yield, shows similar variation to sesamin, as the two components were obtained from exactly the same conditions. Generally, sesame oil yield increased with increase in pressure, and extraction time. The total lignan content is shown on Figure 2-3, where it is clearly presented that higher lignan content is recovered at the initial stage of the process. Even, at the beginning of the first 15 minutes, highest total lignan value was obtained. The total lignan obtained at 400 bar and 15 minute is almost double to that of 200 bar at 15 minute. This shows that pressure has strong influence on the extraction of sesame oil lignans in supercritical carbon dioxide condition.

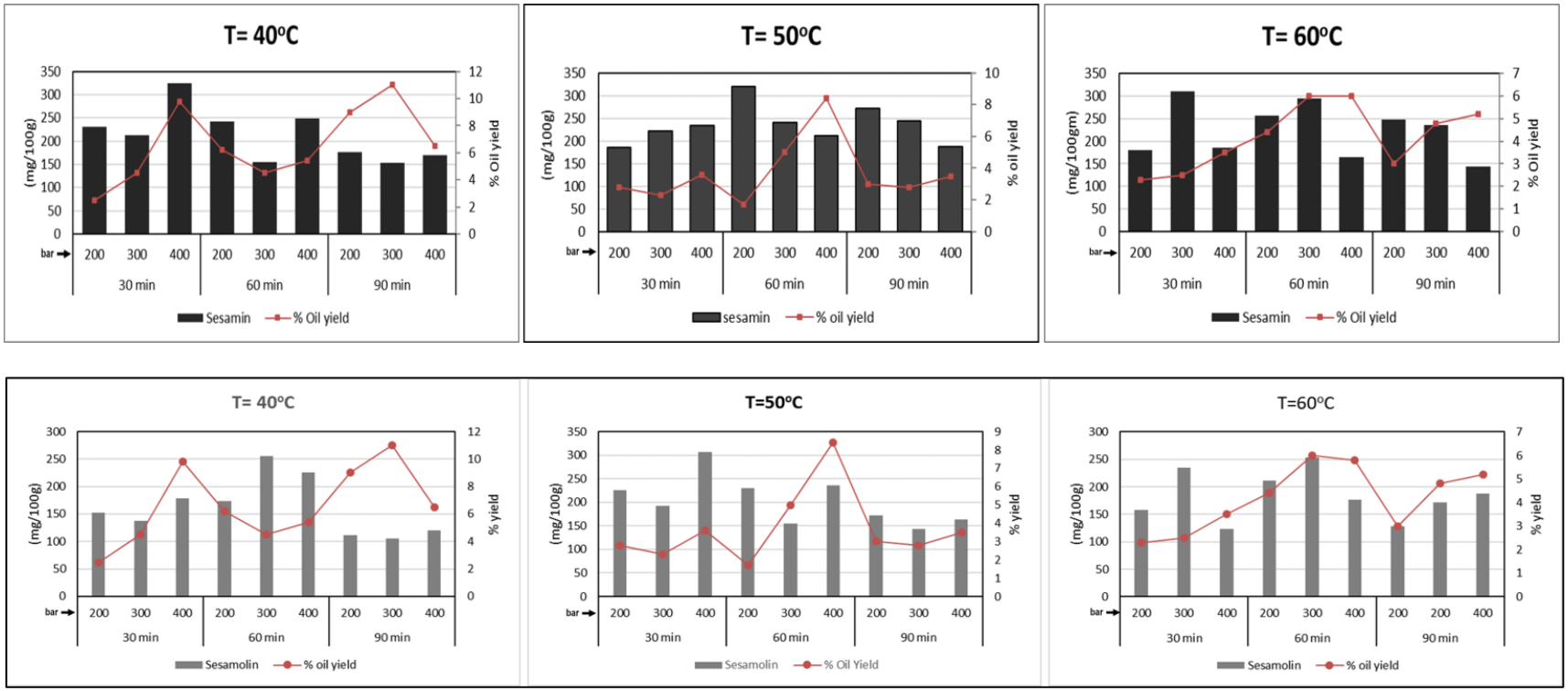



Figure 2- 2 Sesame oil lignan extraction using supercritical $CO_2$ at different temperature, pressure and time

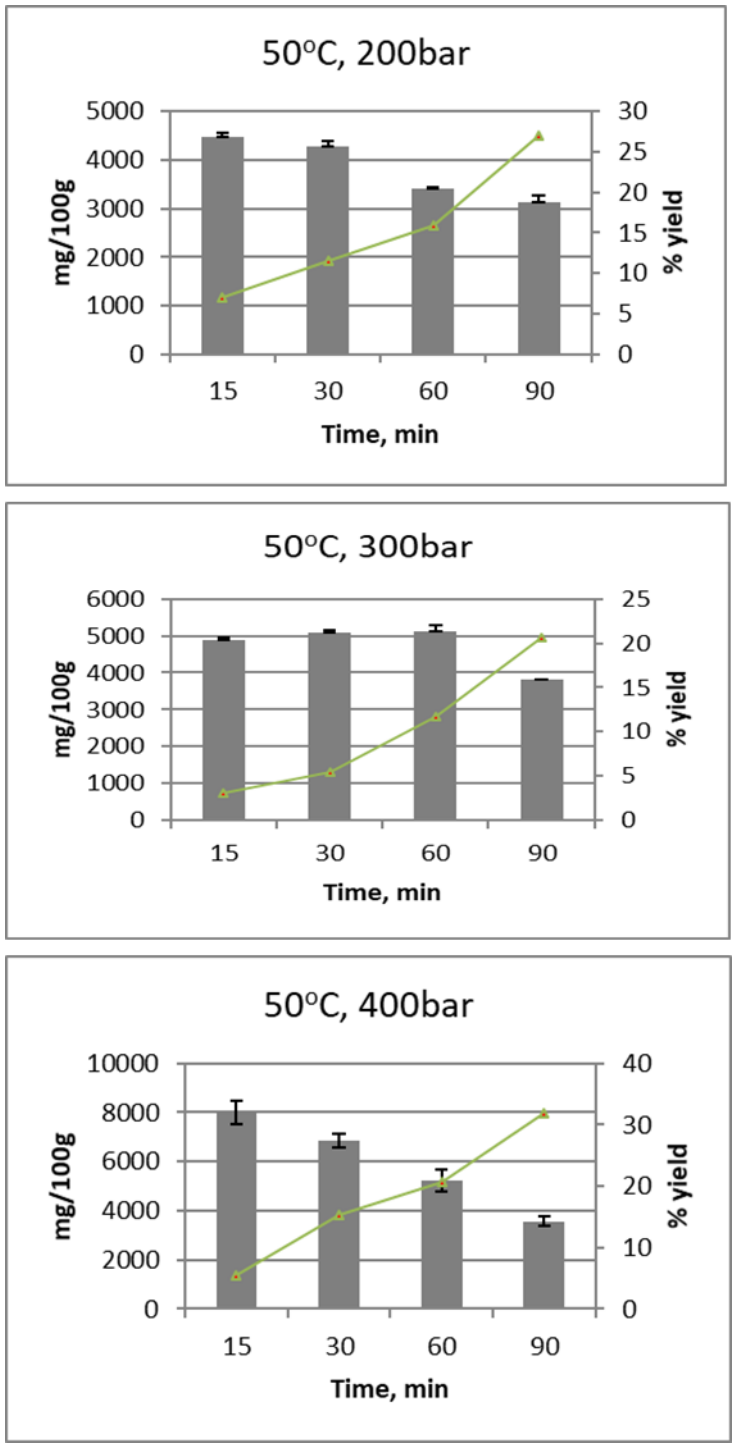



Figure 2- 3 Total lignan content, extracted at, (a) 50 $^{o}$C, 200 bar, (b) 50 $^{o}$C, 300 bar, and (c) 50 $^{o}$C, 400 bar, using supercritical $CO_2$

### 2.3.2. Lignan transformation under supercritical carbon dioxide

After sesame oil extraction using supercritical carbon dioxide, the lignan content is analysed using HPLC analysis. Then, sesame oil lignan transformation was performed to bring about changes on the lignan composition of the oil. The purpose of lignan transformation was to convert sesamolin to sesamol, a more bioactive component. The HPLC analysis clearly revealed the peak size and area differences of sesame oil lignans before and after conversions with respect to the different reaction times. Initially, the untreated sesame oil lignan composition with regard to the contents of sesamin, sesamolin, and sesamol was 52.85%, 38.91%, and 3.66% respectively. At the beginning, neither samin nor sesaminol were detected in the untreated sesame oil. However, after being treated for about 60 minutes, in supercritical $CO_2$ at 80 $^oC$ and 160 bar, as shown in Figure 2-4, the area percent of sesamin, sesamolin, sesamol, and sesaminol was 47.67%, 15.41, and 30.82%, and 0.25% respectively. Sesamolin, was significantly converted to sesamol at this condition. However, the content of sesaminol was very small as compared to sesamol. As shown in (figure 2-5) significant changes in the area percent composition of sesame oil lignans was observed at 100$^oC$, 180bar. For this condition, after a reaction time of 30 minutes the area percent of sesamin, sesamolin, sesamol, and sesaminol was found to be 51.85%, 8.1%, 28.5%, and 3.3% respectively. Here, sesamolin was even further transformed to other lignans, such as sesamol, sesaminol and small amount of samin was detected. Sesamin showed almost no variation in this condition. This is an indication that sesamin have strong resistance for oxidation, even at elevated temperatures.

However, after reaction times of 60 minutes, both sesamin and sesamol showed sign of degradations. That might have happened due to the continuous exposure of lignans at extreme conditions. The transformation of sesamolin was also checked at even higher temperature ($120^0$C), which is the maximum operating temperature of Amberlyst-15. As shown in (Figure 2-6), the lignan transformation was achieved even at faster rate. In just 20 minutes, under supercritical $CO_2$ of $120^0$C and 200bar, the area percent of sesamin, sesamolin, sesamol, sesaminol, and samin was found to be 49.23%, 3.84%, 34.44%, 4.31%, and 0.85% respectively. As the reaction temperature is increased the lignan transformation was also increased proportionally. However, sesamol was dramatically degraded along with increase in reaction time. In this case, the maximum samin and sesaminol content was found to be 3.58% and 8.31% respectively at reaction time of 30 minutes. At even longer reaction time (60 minutes) sesamolin was almost completely transformed to other lignans, however the produced lignans such as sesamol, and samin were significantly reduced. It can be clearly seen that almost in all reaction conditions sesamin content don't show significant variations. This indicates that the catalytic reaction is specifically targeted on sesamolin only. This is very important situation as specified on Table 5, the antioxidant activity of sesamin is greater than sesamolin, hence the overall antioxidant property of sesame oil is improved.

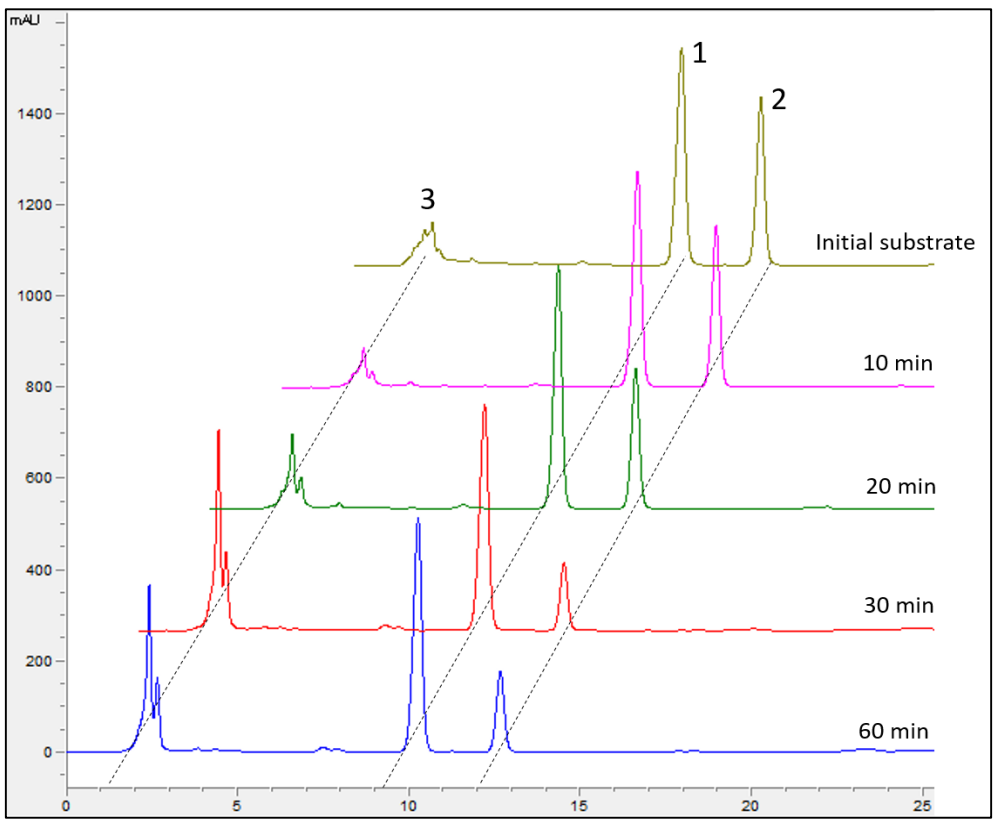



Figure 2- 4 HPLC chromatogram of sesamolin conversion in supercritical carbon dioxide at 80ºC, 160bar. (1) sesamin, (2) sesamolin, (3) sesamol, and (4) sesaminol.

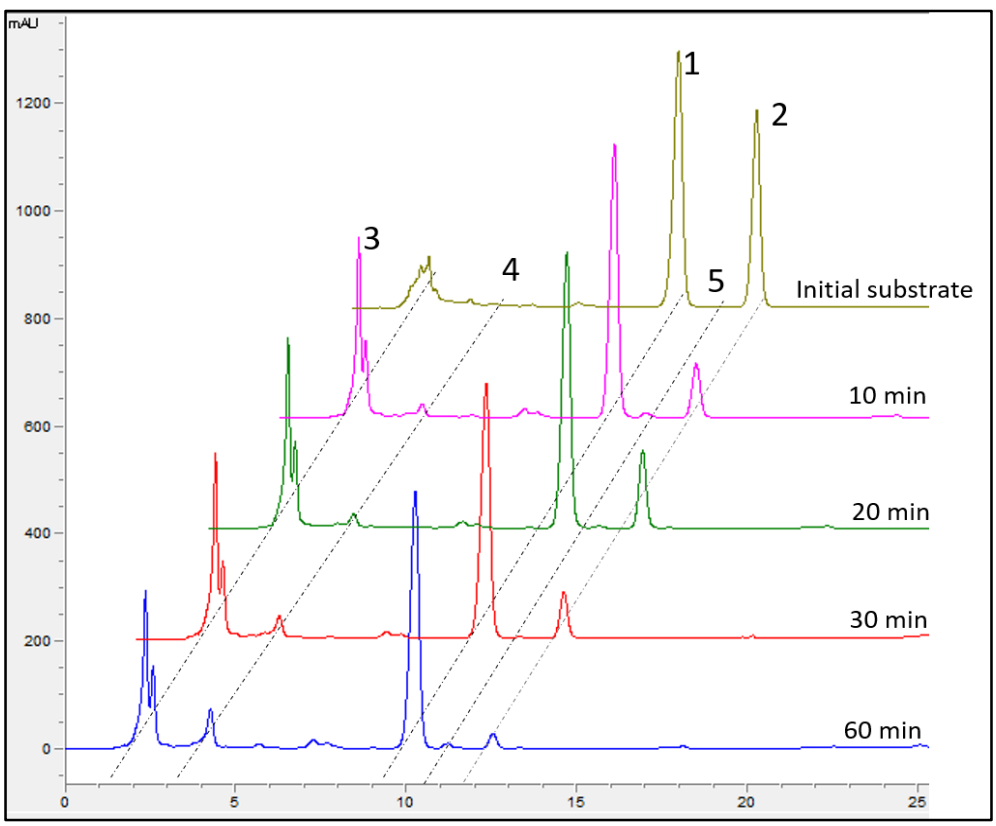



Figure 2- 5 HPLC chromatogram of lignan conversion in supercritical carbon dioxide at 100°C and 180bar. (1) sesamin, (2) sesamolin, (3) sesamol, (4) sesaminol, and (5) samin.

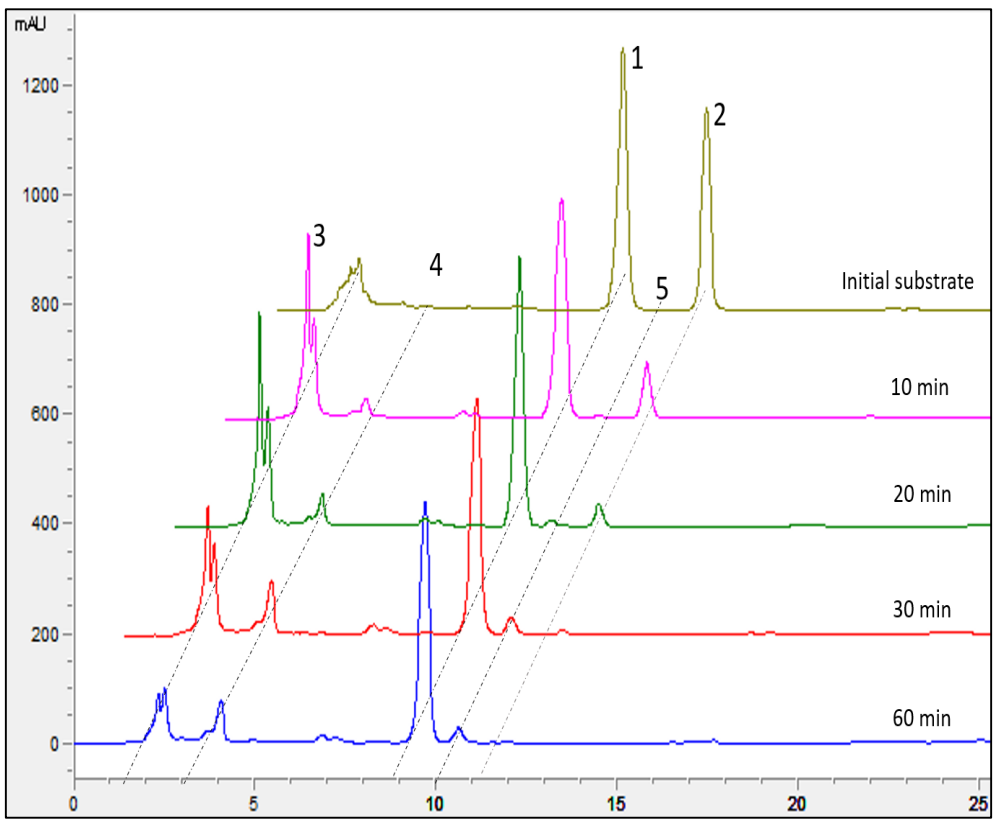



Figure 2- 6 HPLC chromatogram of sesamolin conversion in supercritical carbon dioxide at 120°C, 200bar. (1) sesamin, (2) sesamolin, (3) sesamol, and (4) sesaminol, and (5) samin.

### 2.4.2.1. The effect of reaction temperature and time on lignan conversion

Effect of reaction time on lignan transformation

Except for sesamin, all the other lignans such as sesamol, and sesamolin showed a significant variation with respect to the changes in reaction time. The lignan conversion under supercritical carbon dioxide at 80$^{o}$C and 160 bar is shown in Figure 2-7. Sesamolin showed significant reduction up to 30 minutes. After that, the rate of sesamolin conversion become slower with increase in reaction time. Nevertheless, the maximum sesamol concentration of 1.06mg/mL was obtained at the maximum reaction time (120 min). Sesamolin concentration was continuously declined to 0.49mg/mL at reaction time of 30 minutes, while sesamol concentration was continuously increased to 0.97mg/mL. Both sesamolin and sesamol don't show significant variations after 30 minutes. Hence for this condition 30 minutes could be considered as optimal reaction time. However, only small amount of samin (0.9µg/mL) and sesaminol (16.6µg/mL) was detected in this reaction condition. Even for the prolonged reaction times, samin content was negligible.

Initially, prolonged reaction time had a positive effect on the formation of sesamol. However, when the reaction temperature was further extended to 100$^{o}$C at a pressure of 180bar, the trend was significantly changed. At a reaction time of 60 minutes, sesamolin concentration was rapidly decreased to 0.14mg/mL as shown on figure 2-8. In which case, sesamolin conversion of 89.6% was attained, while sesamol percent yield was declined to 31.5%. After 60 minutes, the decrease in sesamol yield could be related to the thermal degradation of sesamol due to the long exposure time or due to the transformation of sesamolin to samin and sesaminol, as it is already

presented (section 1.4.2), that even small quantities of samin and sesaminol start to appear in the chromatograms (Figure 2-5) at a reaction time of 60 minutes.

Finally, the catalytic lignan transformation was performed at 120ºC, which is the maximum operating temperature of Amberlyst-15. In just 20 minutes, sesamolin concentration was reduced to 0.19mg/mL, which is 85.6% conversion, and maximum sesamol concentration of 0.86mg/mL, which accounts for 65.3% of yield was achieved (figure 2-9). However, sesamol content was sharply decreased as reaction time increased. The maximum samin content of 0.42mg/mL was recorded in this condition at 120minutes. Probably, the decrease in the yield of sesamol could be due to the conversion of sesamolin to samin and sesaminol instead of sesamol. This is also supported by the HPLC analysis of lignan composition (Figure 2-6).

Generally, after further extending the reaction time to 120 min, the yield of sesamol was almost completely diminished. Similarly, sesamolin content was significantly reduced. The decrease in lignan content could due to the destruction of both sesamolin and sesamol because of the extreme temperature effects and prolong exposure time. The trend of lignan transformation in Sc-$CO_2$ for temperatures of 80 and 100 ºC, seems to be in agreement with the lignan transformation in toluene. Hence, the lignan transformation mechanism proposed for general acidic condition [40] could also be considered for the Sc-$CO_2$ lignan transformation as well, since sulphuric acid is source of acidic media in both cases. However, at the maximum temperature (120 ºC) the content of samin showed gradual increment and that could be related to the change in lignan transformation mechanism at higher temperatures or due to the generation of samin from other lignan components.

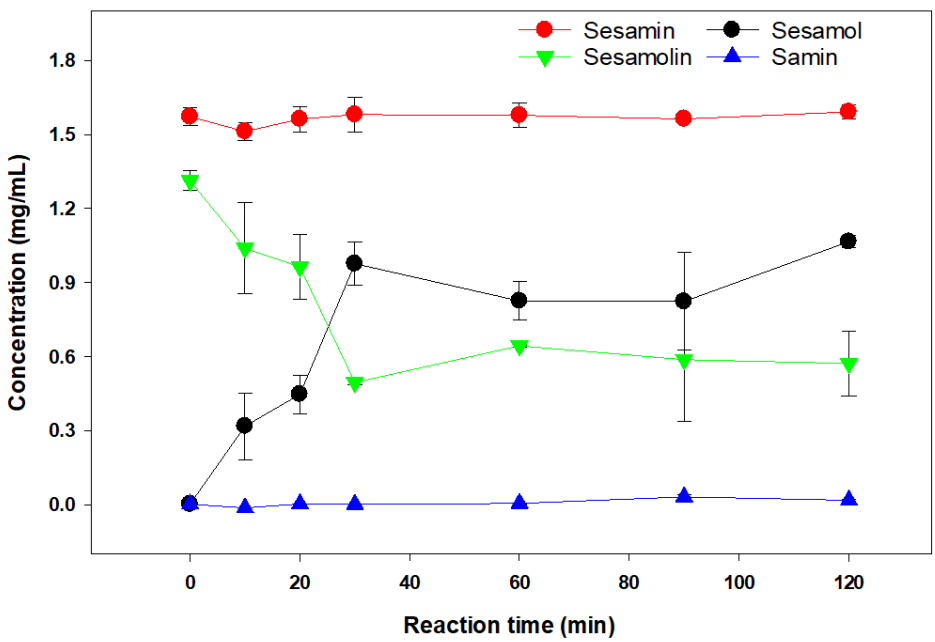



Figure 2- 7 Sesamolin transformation in supercritical carbon dioxide at 80ºC and 160bar.

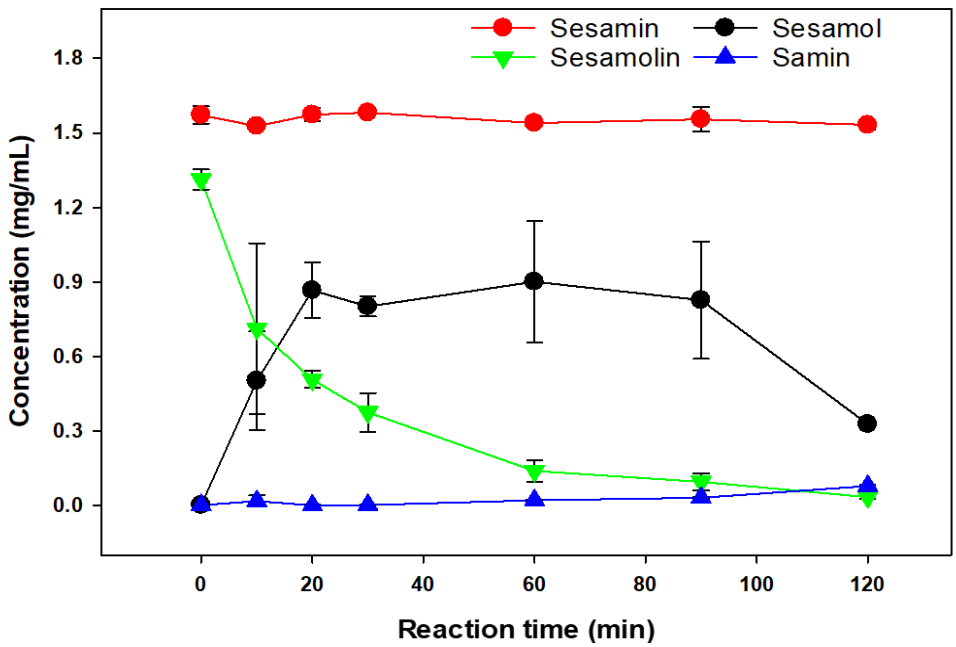



Figure 2- 8 Sesamolin transformation in supercritical carbon dioxide at 100°C and 180bar.

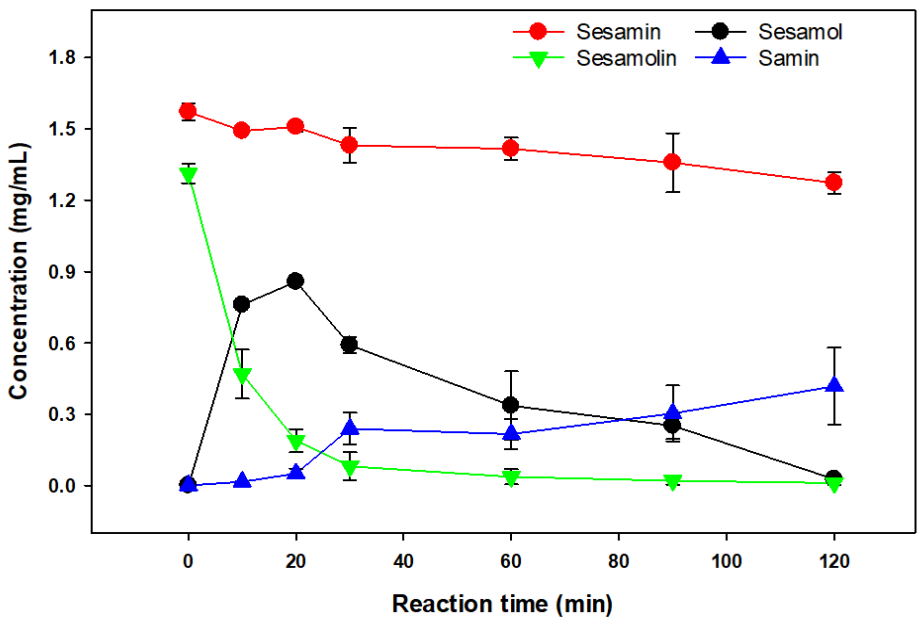



Figure 2- 9 Sesamolin transformation in supercritical carbon dioxide at 120ºC and 200bar.

For this reason, further investigation of lignan transformation mechanism in supercritical carbon dioxide should be performed to better understand the mechanism.

## Effect of reaction temperature on lignan transformation

The effect of reaction temperature (80$^{o}$C, 100$^{o}$C, and 120$^{o}$C) on sesame oil lignan conversion was analysed. The percentage conversion and percentage yield of sesame lignan for reaction time of 10min and 20min, is shown on (Figure 2-10, A and B) respectively. At reaction time of 20 minute, sesamol yield was increased from 26% to 65.9% for reaction temperature of 80$^{o}$C and 100$^{o}$C respectively. However, sesamol yield did not increase at higher temperature. For example, almost the same yield (65%) was obtained even if the temperature was further raised to 120$^{o}$C. Likewise, sesamolin conversion was increased from 15.87% at 80$^{o}$C to 61.38% at 100$^{o}$C (Figure 2-10, B). However, at higher temperature more sesamolin was converted to sesamol and samin. For example, sesamolin conversion was increased significantly from 61.38% at 100$^{o}$C to 85.6% at 120$^{o}$C. This shows that, higher temperature favours the conversion of sesamolin. Surprisingly, in all this conditions sesamin didn't show variations with temperature. It is kept constant with the initial value almost throughout the reaction time, at temperatures of 80$^{o}$C, and 100$^{o}$C.

Generally, for all the reaction times, sesamol yield was greatly increased with the increase in reaction temperatures. Similarly, sesamolin was also greatly transformed to sesamol and samin. The maximum sesamol yield of 80.07% and maximum conversion of sesamolin of 98.1% was recorded at 80$^{o}$C, 120 min and 120$^{o}$C, 90min respectively (Figure 2-12).

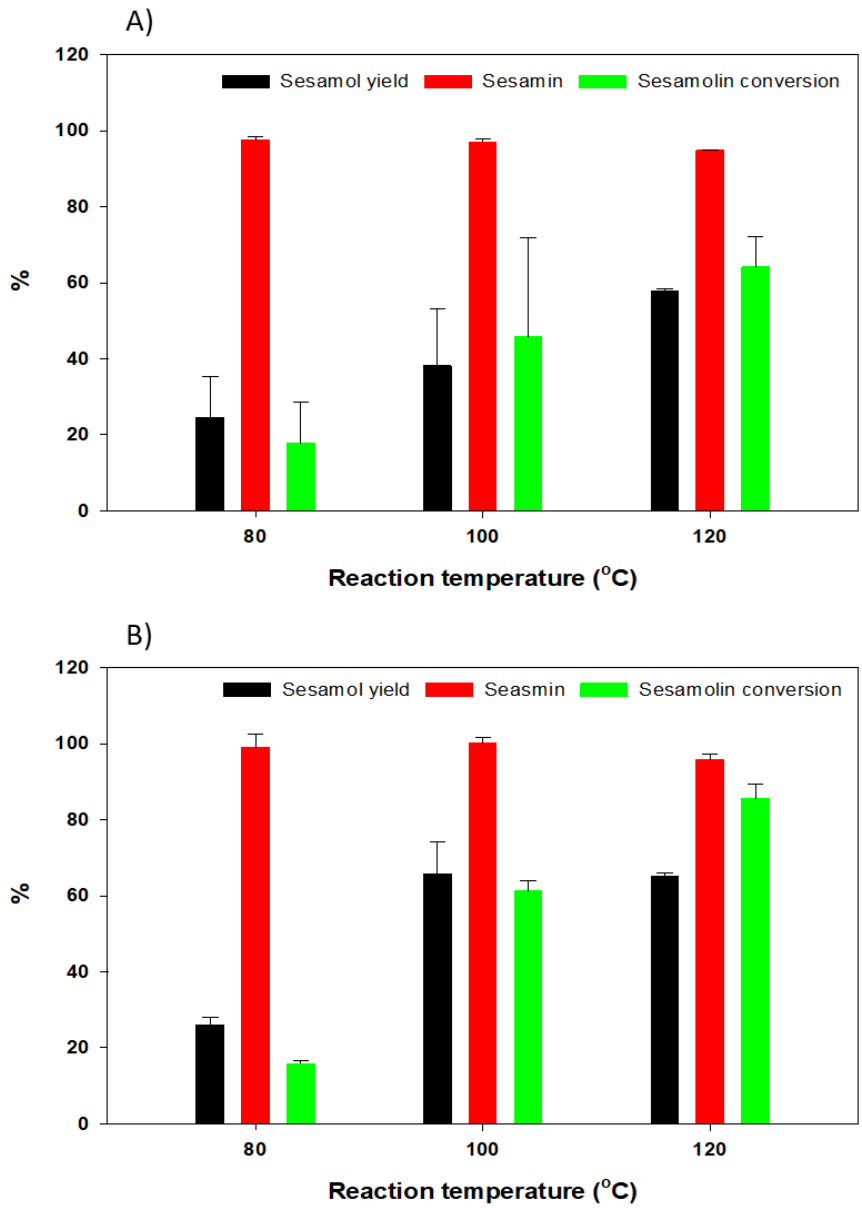



Figure 2- 10 Effect of reaction temperature on lignan conversion under supercritical $CO_2$ condition A) reaction time of 10 min, and B) 20 minutes.

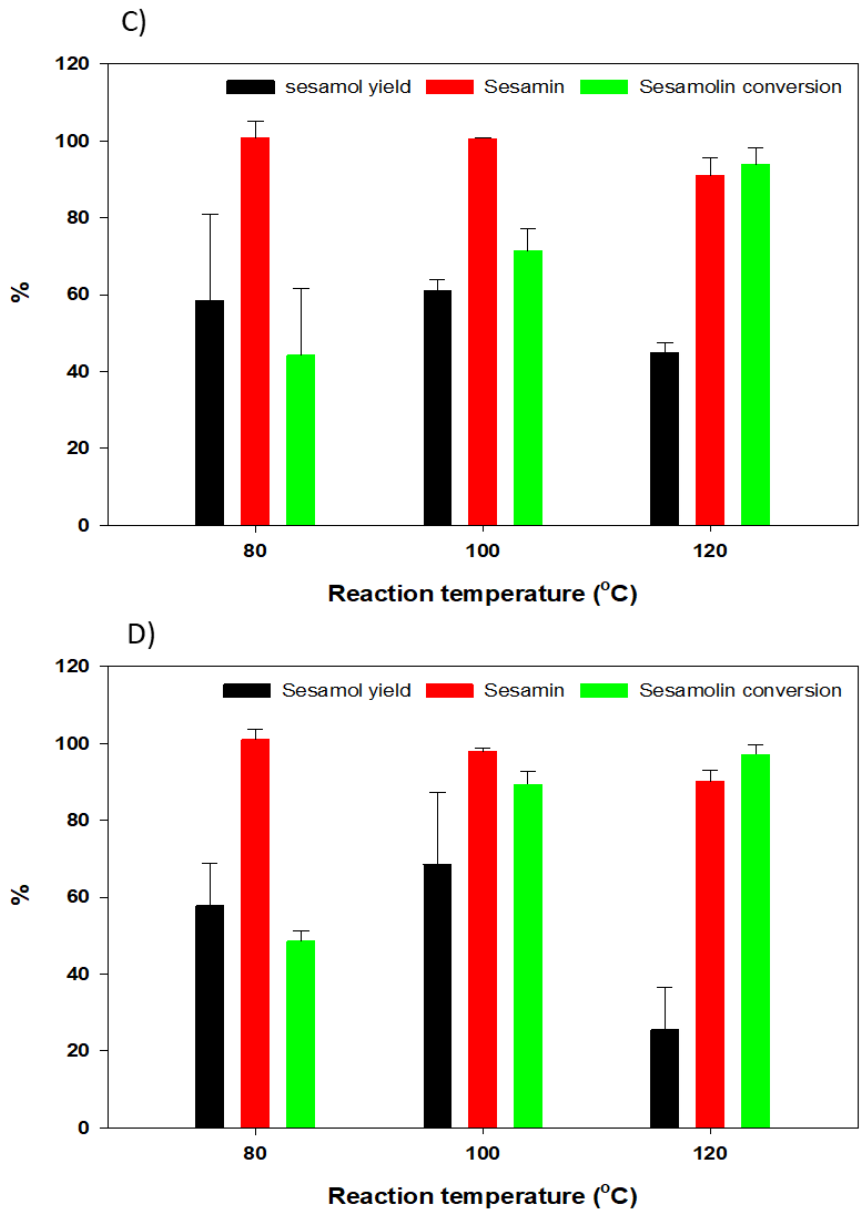



Figure 2- 11Effect of reaction temperature on lignan conversion under supercritical $CO_2$ condition C) 30 min of reaction time, and D) 60 minute.

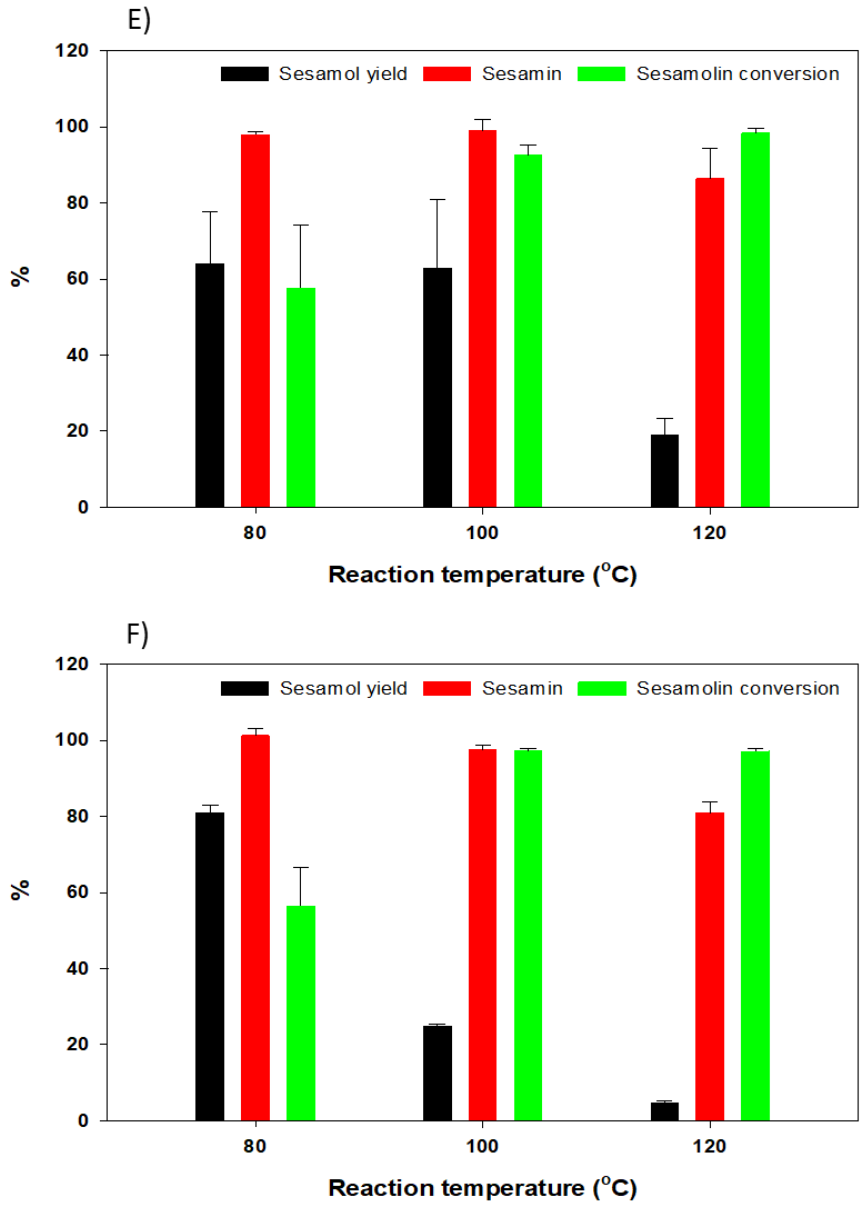



Figure 2- 12 Effect of reaction temperature on lignan conversion under supercritical $CO_2$ condition E) 90 min, reaction time, and F) 120 min.

2.3.3. Sesame oil FTIR characterization

Figure 2-13, reveals FTIR spectra of sesame oil before and after lignan transformation is performed. Both FTIR spectra showed the resemblance of sesame oil, indicating there is no significant alterations on the oil after reaction. The peaks at 2930 and 2853 were coming from asymmetric and symmetric stretching vibrations of methylene (-CH2). The carbonyl (C=O) stretching vibration was observed at 1750 $cm^{-1}$, while the peak at 1654 is from C=C stretching vibration. The bending vibrations of methylene and methyl were observed at wavenumbers of 1460 and 1376 $cm^{-1}$, respectively. The peaks at regions of 1237, 1150, 1080 were from C-O vibrations. While, peaks at 996 and 850 were due to bending out of plane vibrations of – HC=CH– (trans) and –HC=CH– (cis), respectively[49]. The FTIR analysis of pure sesamol is presented in Table 6. Here, the peaks in the range of mid-1600, are indications for the existence of phenyl structures. Additionally, in the graph a small increment of peak is noticed after reaction in the range of 1500 to 1650$cm^{-1}$ also shows the presence of phenyl functional group which is the characteristic of sesame lignan.

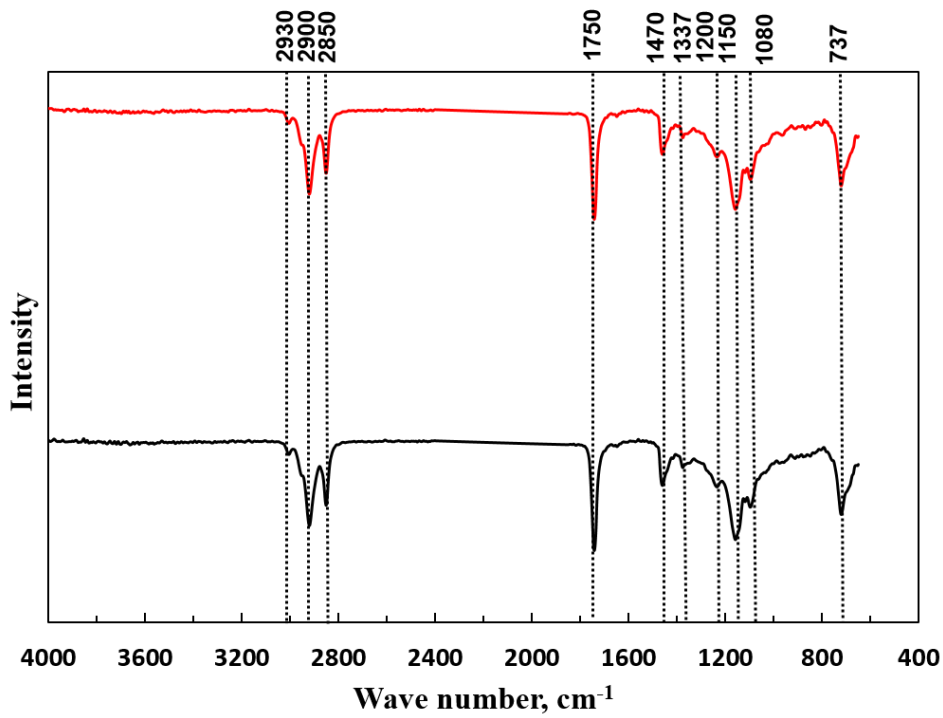



Figure 2- 13 FT-IR analysis of sesame oil (red line) after reaction and (black line) before reaction.

Table 6 Peak Assignments for sesamol [50]

| No | **Peak ($cm^{-1}$)** | **Functional group** | **Remarks** |
|---|---|---|---|
| 1 | 3401 | –OH | OH of phenolic O–H |
| 2 | 3007 | –CH | Unsaturated–CH |
| | 2896 | –CH | Saturated–CH |
| 3 | 2777 | $–CH_2$ | Symmetric stretching |
| 4 | 1637 | Phenyl | Phenyl skeletal frequency |
| 5 | 1504 | Phenyl | Out-of-plane |
| | 1489 | CH | Bending |
| | 1471 | $–CH_2$ | $CH_2$ bending (1480 cm−1) |
| 6 | 1397 | Methyl | Methyl symmetric bending |
| 7 | 1274 | C–O | C–O of phenolic |
| 8 | 1185 | C–H | In-plane bending of aromatic C–H |
| 9 | 1125 | C–O–C | Symmetric stretching |
| 10 | 1093 | –CH | Probably in-plane bending of phenolic CH enhanced by polar substituent |
| 11 | 1039 | =C–O–C | Symmetric stretching |
| 12 | 944 | C–O | Most characteristic for methylenedioxy |
| | 929 | | (927 $cm^{-1}$), probably related to C–O stretching |
| 13 | 835 | –CH | CH of two adjacent hydrogens at 3,4 positions |
| 14 | 815 | –CH | Out-of-plane CH bending bands |
| | 796 | | suggest adjacent hydrogen atoms |
| 15 | 766 | | Di-substitution of phenyl |
| 16 | 714 | C–O–C | Weak bands, sometimes absent |
| | 611 | | |

# Chapter 3. Water soluble bioactive component recovery

## 3.1. Introduction

During sesame oil production, more than 50% by weight of the dry seed is composed of solid residue (also known as sesame meal), which is often considered as a byproduct. Sesame meal like sesame oil contains highly nutritious components such as phenols, flavonoids and polar lignans like sesaminol, sesaminol-glycosides, sesamolinol and pinoresinol (shown on Figure 3-1)[13,51]. However, during oil extraction, these components are left with the solid residue because of their polar nature. These polar compounds have physiological functions in our body, such as antioxidants, anti-inflammatory and anticancer agents[52–55]. However, since the cake is usually considered as a byproduct in some countries, it's used as livestock feed hence the essential components are often lost before recovery.

Sesame seed meal is also very rich in dietary components, protein, carbohydrates, and fibrous material[12]. In addition to the aforementioned components, the water-soluble (glycosylated) lignan components are mainly concentrated in the defatted sesame meal (DSM)[56,57]. Oil-soluble lignans, such as sesamin, sesamolin, sesamol, and tocopherols, are known for their strong resistance to oxidative deterioration[58,59]. Moreover, DSM is also a source of different phytochemicals, phenolic compounds, polyphenols, and water-soluble furofuran lignans [60–62]. These compounds are multi-functional in the human body, where they lower cholesterol[63], reduce inflammation and atherosclerosis[52,55], and act as antioxidants[45,53,64] and anti-inflammatory agents[52,55]. In addition, some nutraceutical components of DSM have reportedly shown anti-proliferative effects

in cancer cells[54,65–68]. For example, Lin et al.[18], and Zhou et al.[60] reported the antioxidant and anti-proliferative activities of sesame seed extracts. However, the recovery of bioactive compounds from defatted sesame seeds is largely performed using conventional extraction methods that employ organic solvents[46,69,70]. Along with the use of environmentally damaging solvents, the other main disadvantages of these conventional extractions are the relatively longer extraction times and higher operational costs due to product purification and solvent recovery[71].

Hydrothermal (HT) extraction, also known as subcritical water extraction, is a widely used technique for extracting bioactive compounds from plant sources[72–74]. The pure water used during this process is usually at high temperatures and pressures that are above the boiling point of water but below its critical point[26]. Compared to conventional extraction, HT-extraction uses green solvents thus posing no environmental or health related problems. It is also considered as one of the techniques for the extraction of wide range of bioactive compounds due to tunability of the polarity[75]. However, sesame seed lignans such as sesaminol and sesamol having moderate polarity are sparingly soluble in water. Hence the extraction of these compounds is favoured by the application of high operating pressures and/or use of non-polar solvents such as $CO_2$ which modifies the dielectric constant of the solvent. Moreover, $CO_2$-assisted HT processes have shown improved product output by enhancing the acid catalysis in HT process[76]. In this regard, $CO_2$ has attracted increasing attention in hydrothermal reactions, such as in the extraction of selected bioactive components from ginseng[77]. It's also reported that $CO_2$ accelerates the

hydrolysis of polysaccharides, producing a major secondary decomposition product, such as 5-Hydroxymethyl furfural (HMF)[78].

Hence, in this work $CO_2$ addition was intended for two main reasons. First, the $CO_2$ at high pressure can be partially solubilized in water to produce a concentrated carbonic acid that can be used as a catalyst for Lewis acid-catalyzed reactions [79]. Second, due to the low dielectric value of a biphasic $H_2O/CO_2$ system, the moderately polar components in DSM can be successfully recovered in the HT-extraction[80]. Therefore, the aim of this research work was to efficiently recover the bioactive compounds from DSM using $CO_2$-assisted HT extraction and to evaluate their antioxidant and anticancer effects on human breast cancer cells.

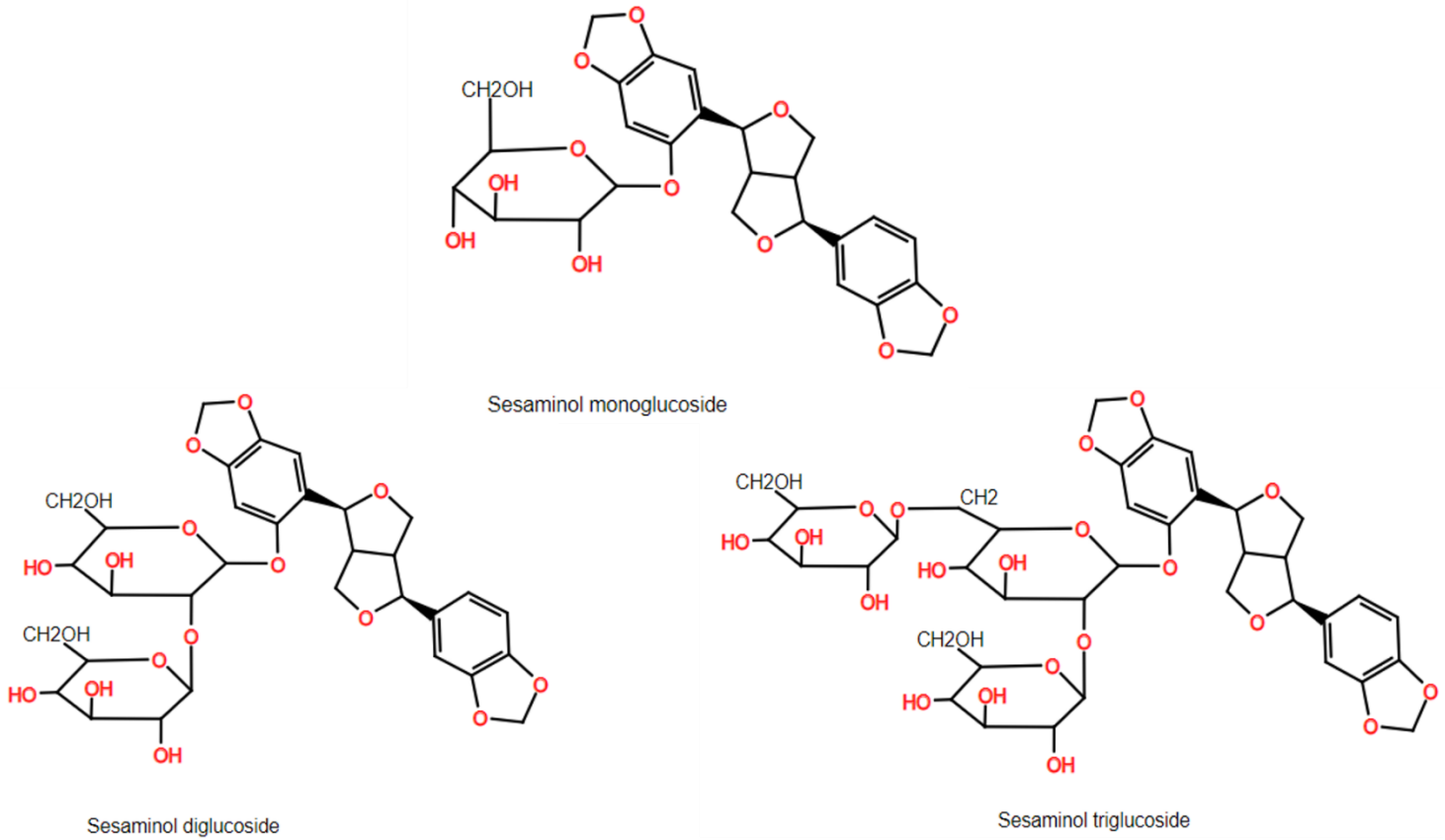



Figure 3- 1 Chemical structure of water soluble sesame lignan components

## 3.2. Experimental

### 3.2.1. Chemicals and reagents

Gallic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS (2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), Folin-Ciocalteu's phenol reagent, and quercetin were purchased from Sigma–Aldrich (Seoul, Korea). Citric acid and aluminum chloride ($AlCl_3$) were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan) and high-performance liquid chromatography (HPLC)-grade water was obtained from J. T. Baker (USA). Sodium sulfate (anhydrous, 98.5% pure), potassium persulfate ($K_2S_2O_8$, 99.0%), sodium carbonate (99.5%), HPLC-grade methanol, and HPLC-grade ethanol were all purchased locally from Samchun Pure Chemicals, Co., Ltd. (Seoul, Korea).

### 3.2.2. Sample pre-treatment

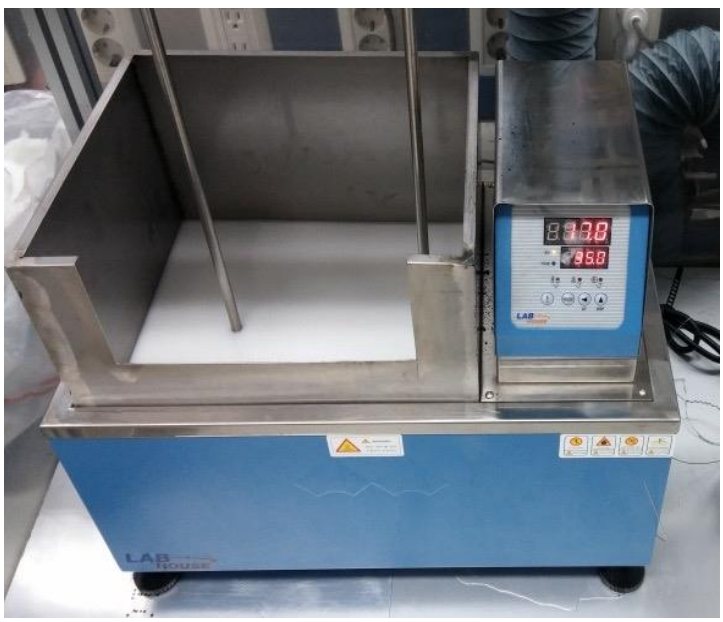
Sesame seeds imported from India were purchased from a local market in Seoul, Korea. The seeds were ground using a grinder (Mill Powder Tech Co., Ltd., Taiwan). Initially, the oil residue was removed from the seed to make an oil free (DSM). The extraction was performed with *n*-hexane in a Soxhlet extractor at 70 ℃ for 12 h. The DSM was then dried in a vacuum oven at 50 ℃, after which it was further ground and sieved to obtain a powder with uniform particle size of 250–500 μm.

## 3.3. Extraction method

### 3.3.1. Hydrothermal extraction

The DSM was put into a 23-mL stainless steel batch reactor (shown on Figure 3-2) (Hanyang Precision CO., LTD, Korea) with a solid to solvent ratio of 1:20 (w/w). For the HT extraction without $CO_2$, the pressure was maintained at 10 MPa, the

temperature ranged from 120 to 180 ℃, and the different extraction times were 20, 40, 60, 90, and 120 min. Based on the operating conditions and reactor volume, approximately 1.05 g of DSM was placed in the reactor and a calculated amount of solvent was added before it was tightly capped. Two $CO_2$-assisted HT (HT-$CO_2$) processes were performed with different DSM to solvent ratios. The HT-$CO_2$ process with a 2:1 ratio (w/w) used 0.375 g of DSM and the solvent comprised 5 g of water + 2.5 g of $CO_2$. The HT-$CO_2$ process with a 4:1 ratio (w/w) used 0.313 g of DSM and a solvent consisting of 5 g of water + 1.25 g of $CO_2$. After loading the DSM and water, liquid $CO_2$ is introduced using a high pressure pump through 1/8" tube connected to a pressure gauge and a valve, which is used for $CO_2$ discharge after the reaction is completed. The reactor was stirred so that it possessed no upper or lower parts. Even though two phases exist, the supercritical $CO_2$ phase extraction rate is insignificant compared to that of the subcritical water phase. In addition, the reactor was positioned horizontally and moved in a back and forth motion at 60 rpm to effectively mix the components. After the extraction time, the reactor was immediately quenched with cold water. The extract was then separated from the solid residue by vacuum filtration on a grade-1 Whatman (110 mm Ø ) filter paper.



Oil bath

Batch reactor

Figure 3- 2 Reaction apparatus, oil bath ans stainless steel batch reactor

### 3.3.2. Conventional extraction

Approximately 7.5 g of DSM was put into a thimble and extracted using 150 mL of different solvents in a Soxhlet extractor. The solvents used were pure water ($H_2O$ Soxhlet), a mixture of 75% ethanol and 25% water (referred to as 75% EtOH), and a mixture of 75% methanol and 25% water (referred to as 75% MeOH). The extractions for each solvent were carried out for 24 h, and then the extract was concentrated by partially evaporating the solvent in a rotary evaporator. All the extracts from both methods were then kept in a freezer at -20 ℃ until use.

## 3.4. Extract analysis and characterization

### 3.4.1. Extraction yield

The extraction yield was determined by placing 2 mL of the extract in a 4-mL vial and heating it at 50 ℃ for 12 h in a vacuum oven, followed by cooling in a desiccator. The dried sample in the vial was weighed to obtain the dry weight of the residues. The yield was then calculated by taking the ratio between the solid residue in the vial and the mass of the initially used dry DSM (Equation 8).

$$\text{Extraction yield } (\%) = 1 - \frac{\text{dry weight of solid residue}}{\text{dry weight of defatted sesame meal}} \times 100\% \qquad (8)$$

### 3.4.2. Bioactivity

Bioactivity is often expressed in terms of total phenol (TP) and total flavonoid (TF). TP content was determined using the Folin-Ciocalteu method[34], and the procedure is briefly explained as follows. First, the standard curve was prepared by a series dilution of 3 mg/mL standard gallic acid in deionized water. The Folin-Ciocalteu reagent (200 μL) is added to 40 μL of the sample extract and then deionized water is added to make a total volume of 1.2 mL. After 10 min, 600 μL of 20% $Na_2CO_3$ was

added to the reagent mixture. The sample was then stored in the absence of light for 2 h, after which the absorbance was read at 720 nm.

The TF content of sesame extract was determined based on the work of Reshma et al.[82] with minor modifications. First, a calibration curve was prepared for a concentration range of 3.125–50 μg/mL by dissolving the appropriate amounts of quercetin standard in methanol. Then, 0.8 mL extracts were mixed with 0.8 mL of 2% $AlCl_3$ in methanol. The mixture was then thoroughly shaken and stored for 10 min in the absence of light before the absorbance was measured at 415 nm.

3.4.3. GC-MS analysis

Gas chromatography-mass spectroscopy (GC-MS) was performed to identify the volatile bioactive compounds. A 7890A GC system (Agilent Technologies, USA) equipped with a 30 m × 0.25 mm × 0.25 μm DB-5ms fused silica capillary column (J&W Scientific, Folsom, CA) and a 5975 mass spectrophotometer (Agilent Technologies, USA) was used. Ultra-high purity helium (99.999%) was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injector temperature was maintained at 250 °C, and the injection volume was 1 μL in splitless mode. The oven temperature was initially held at 40 ℃ for 2 min, then the temperature was raised to 300 ℃ at a rate of 10 ℃/min and held for 15 min. The total run time was 46 min. The temperatures of the MS ion source and transfer line were held at 230 ℃ and 250 ℃, respectively. Mass spectra were obtained with a scan range of *m/z* 50–650 and an electron impact ionization energy of 70 eV.

3.4.4. Antioxidant activity

The antioxidant activity of bioactive compounds is often tested using DPPH radical scavenging, ABTS radical scavenging, and ferric reducing antioxidant power

(FRAP) assays among others. We employed the first two assays (DPPH and ABTS) because of their wide applicability.

3.4.4.1. DPPH radical scavenging capacity

The DPPH radical was first generated by dissolving DPPH powder in methanol to make a 1 mM solution. A 2 mM Trolox solution (5 mg/mL) was also prepared as a positive control. The Trolox solution (100 μL) was added to 1 mL of DPPH and after 30 min the absorbance was measured at 517 nm, during which the solution color changed from purple to light yellow. This color change is an indication of DPPH radical scavenging. Similarly, 100 μL of DSM extract was added to 1 mL of the DPPH radical solution and the scavenging capacity was then compared against that of the Trolox to calculate the scavenging capacity percentage (Equation 9).

$$\text{DPPH radical scavenging capacity (\%)} = \left[\frac{\{A_{DPPH} - (A_S - A_{Sc})\}}{A_{DPPH}}\right] \text{x } 100\% \qquad (9)$$

where $A_{DPPH}$, $A_S$, and $A_{Sc}$ refer to the absorbance of the DPPH solution, sample, and sample control, respectively.

3.4.4.2. ABTS radical inhibition capacity

A 7 mM ABTS solution was prepared by dissolving 38.4 mg of ABTS in 10 mL of deionized water. Another solution of 6.62 mg of potassium persulfate in 10 mL of methanol was also prepared in a different flask. Both solutions were mixed and stored for 16 h in the absence of light to produce the $ABTS^+$ radical solution. DSM extracts (100 μL) were added to 1.0 mL of the $ABTS^+$ radical solution and stored in the dark for 10 min. The absorbance was then read at 734 nm and compared against that of the positive control (Trolox solution). The absorbance reading was converted to concentration according to an improved ABTS assay determination technique by Nicoletta et al.[36], as shown in Equation 10.

$$\text{ABTs radical scavenging capacity (\%)} = \left[\frac{\{\text{Abs}_{\text{control}} - \text{Abs}_{\text{Sample}}\}}{\text{Abs}_{\text{control}}}\right] \text{x}100\% \quad (10)$$

Here, $\text{Abs}_{\text{control}}$ and $\text{Abs}_{\text{sample}}$ are the absorbance of the control and sample, respectively.

### 3.4.5. In vitro anticancer tests

Human breast cancer cell lines (MDA-MB-231) and human dermal fibroblast (HDF) cells were purchased from American Type Culture Collection (ATCC, USA) and cultured in a complete medium (Dulbecco's modified Eagle's medium (Gibco, USA)) that was supplemented with 10% (v/v) fetal bovine serum (Gibco) and 1% (v/v) penicillin/streptomycin (Gibco). The cells were incubated at 37 ℃ with 5% $CO_2$. The MDA-MB-231 and HDF cells were collected using trypsin-EDTA and then counted and resuspended in the complete medium. For the cell viability assay, $1\times10^3$ cells were seeded per well in a 24-well microplate. For the Live/Dead assay, $1\times10^4$ cells were seeded per well in a 96-well microplate.

#### 3.4.5.1. Cell viability and cytotoxicity assay

Cell viability was evaluated using the EZ-CYTOX Cell Viability Assay Kit (DAEILLAB SERVICE, Korea) according to the manufacturer's protocol. Briefly, the MDA-MB-231 cancer cells and normal cells (HDF cells) were cultured with various concentrations (0, 0.625, 1.25, 2.5, and 5 mg/mL) of the HT, HT-$CO_2$, and conventional (75% MeOH) extracts for 24 h each, then 10% WST reagent solution (v/v) was added to each well and incubated at 37 ℃ for an additional 2 h. The absorbance was measured at 450 nm using a plate reader. Cell viability was calculated as the percentage of viable cells relative to the untreated group (n=5 per group). Viable and dead cells were detected by fluorescein diacetate and ethidium

bromide, respectively. The MDA-MB-231 and HDF cells were treated with extracts and incubated at 37 ℃ for 24 h. After the reaction, FDA/EB solution was added to each well. They were incubated for 5 min at 37 ℃ and then rinsed with PBS. The stained cells were imaged using a fluorescence microscope (IX71 inverted microscope, Olympus).

## 3.5. Result and discussion

### 3.5.1. Extraction yield

The extraction yield shows the amount of hydrolyzate components dissolved in water and was calculated according to Equation 8. It is well known that increased extraction temperature leads to increased extraction with an increase in the risk of component degradation. The maximum extraction yield was 60.2% (w/w), which was obtained at 180 ℃ over 120 min via the HT-$CO_2$ (2:1, w/w) extraction (Figure 3-3). The minimum yield was 23.9% and it was obtained by the HT extraction that proceeded for 120 min at 120 ℃ (HT-120).

In the conventional extraction (Table 7), a yield of 15.3% was obtained by extraction with 75% MeOH for 24 h. The HT extraction yield continuously increased from 7.1% to 23.9% at a temperature of 120 ℃ and from 20.4% to 42.2% at 150 ℃. In fact, at 180 ℃ a sharp increase in extraction yield was obtained after only 20 min (Figure 3-3), which was not seen when temperatures of 120 and 150 ℃ were used. However, at a higher temperature (180 ℃) the extraction yield varied slightly with an increase in extraction time. To determine if variations occurred upon changing temperature, we compared extreme extraction conditions at 180 and 120 ℃.

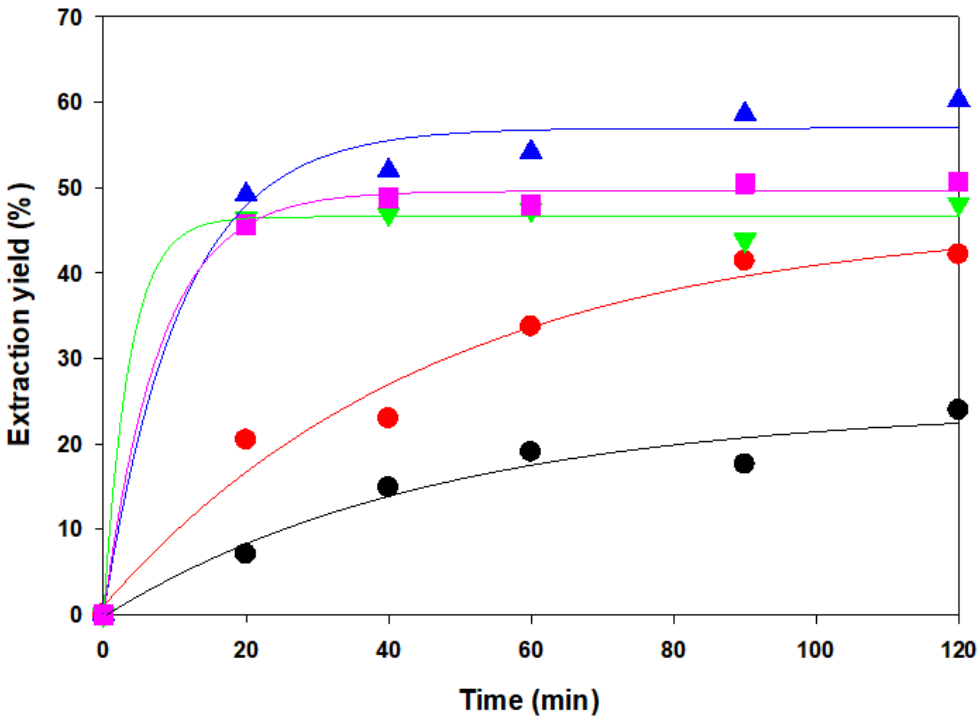



Figure 3- 3 DSM extraction yield with time for hydrothermal (HT) extraction and HT extraction with $CO_2$ addition (HT-$CO_2$). Black circles represent HT extraction performed at 120 ℃ (HT-120) and 10 MPa, red circles represent HT-150 (10 MPa), down green triangles represent HT-180 (10 MPa), blue triangles represent HT-$CO_2$ (2:1, w/w) performed at 180 ℃ and 10 MPa, and purple squares represent HT-$CO_2$ (4:1, w/w) performed at 180 ℃ and 6 MPa.

Table 7 Bioactive components, total phenol (TF) and total flavonoid (TF), and antioxidant activity in terms of $IC_{50}$ values of hydrothermal (HT) extraction, conventional extraction, and water soxhlet extractions.

| | Temperature (°C) | Time (min) | TP (mg GAE/g dry DSM) | TF (mg Quercetin/g dry DSM) | $IC_{50}$ (equiv.Trolox/g dry DSM) |
|---|---|---|---|---|---|
| HT-Extraction [a] | 120 | 20 | 0.92±0.08 | 0.51±0.12 | 12.41±0.51 |
| | | 40 | 1.14±0.11 | 0.59±0.41 | 14.19±1.12 |
| | | 60 | 2.87±0.44 | 2.56±0.81 | 10.81±2.43 |
| | | 90 | 1.67±0.12 | 1.26±0.24 | 11.77±2.84 |
| | | 120 | 2.72±0.54 | 0.34±0.09 | 8.15±1.18 |
| HT-Extraction [a] | 150 | 20 | 2.73±0.65 | 1.81±0.31 | 11.45±0.41 |
| | | 40 | 2.86±0.94 | 1.59±0.43 | 8.6±0.71 |
| | | 60 | 4.18±0.71 | 2.57±0.45 | 4.42±0.85 |
| | | 90 | 5.52±1.11 | 3.16±0.86 | 5.63±1.84 |
| | | 120 | 4.32±0.96 | 1.88±0.32 | 4.98±0.84 |
| HT-Extraction [a] | 180 | 20 | 6.31±1.4 | 3.05±0.82 | 2.15±0.85 |
| | | 40 | 11.23±1.1 | 4.08±1.11 | 1.91±0.42 |
| | | 60 | 12.13±1.1 | 3.48±0.55 | 1.56±0.18 |
| | | 90 | 9.66±1.09 | 4.86±0.52 | 1.46±0.15 |

| | | | | | |
|---|---|---|---|---|---|
| | | 120 | 7.58±1.2 | 3.13±1.43 | 1.55±0.65 |
| | | 20 | 10.76±1.56 | 2.63±0.78 | 2.04±0.23 |
| | | 40 | 12.87±2.06 | 3.95±0.58 | 1.82±0.30 |
| HT-$CO_2$ (2:1, w/w) [a] | 180 | 60 | 15.94±1.56 | 6.07±0.81 | 1.29±0.31 |
| ($x_{CO_2}$ = 0.0113) | | 90 | 11.94±1.64 | 5.37±0.76 | 1.39±0.26 |
| ($y_{CO_2}$ = 0.9011) | | 120 | 13.56±2.24 | 5.95±1.03 | 1.31±0.24 |
| | | 20 | 9.43±1.35 | 0.77±0.11 | 2.10±0.43 |
| HT-$CO_2$ (4:1, w/w) [a] | 180 | 40 | 12.95±2.52 | 1.43±0.55 | 1.44±0.11 |
| ($x_{CO_2}$ = 0.0113) | | 60 | 14.08±1.81 | 2.56±0.31 | 0.97±0.21 |
| ($y_{CO_2}$ = 0.8230) | | 90 | 12.90±1.21 | 2.71±0.61 | 1.07±0.12 |
| | | 120 | 11.05±1.82 | 3.97±0.45 | 1.35±0.19 |
| Conventional extraction | | | | | |
| 75% MeOH [b] | | 24 h | 2.81±0.46 | 3.02±0.12 | 9.64±0.21 |
| 75% EtOH [b] | | 24 h | 1.31±0.52 | 2.87±0.26 | 11.26±0.92 |
| Water-soxhlet [b] | | 24 h | 3.86±0.2 | 1.57±0.37 | 7.23±0.61 |

TP = total phenolic; TF = total flavonoids; $IC_{50}$ = inhibition concentration reduced by 50%

[a] The extraction was performed using the $H_2O$: $CO_2$ mixture in 2:1 and 4:1 ratios at 10MPa and 6MPa, respectively.

[b] Extraction was performed in a Soxhlet extractor using a 75% methanol-water mixture, 75% ethanol-water mixture, or pure deionized water at 1 atm.

$x_{CO_2}$ is the mole fraction of $CO_2$ in water-rich liquid phase and $y_{CO_2}$ is the mole fraction of $CO_2$ in gaseous $CO_2$-rich phase

The maximum extraction yield of 48.2% obtained at 180 ℃ without $CO_2$ addition was more than double that obtained at 120 ℃ (23.9%). This shows that temperature has a more profound effect on extraction yield than extraction time does. However, higher temperatures often increase the risk of bioactive compound degradation. Hence, the experiment was performed at 180 ℃ with water to $CO_2$ ratios of 4:1 and 2:1, where the extraction yield was further increased by approximately 5.3% and 25%, respectively, compared to that of the HT-180 extraction without $CO_2$. The addition of $CO_2$ may have increased the extraction yield by creating conducive conditions (due to $CO_2$ dissociation in water) that facilitated the hydrolysis reaction. This would consequently ramp up the mass transfer of components from the solid residue to the aqueous media.

The extraction yields of conventional extraction were 15.3% and 13.5% for 75% MeOH and 75% EtOH, respectively (Table 7). The yields of conventional extractions were slightly lower than that of the Soxhlet water extraction yield (19.4%). Even though the extraction periods of both the conventional extractions and Soxhlet water extraction were 24 h, the yields were much lower than that of the HT extraction. This is likely due to the very low temperature applied in the Soxhlet extractions. In the $CO_2$-assisted HT extraction, a higher extraction yield was obtained when an $H_2O$:$CO_2$ ratio of 2:1 (w/w) was used at 180 ℃ and 10 MPa. Generally, at lower temperatures the yield obtained from HT extraction without $CO_2$ was comparable to that of conventional extractions.

### 3.5.2. Total phenol and total flavonoid contents

The total phenol (TP) content was enhanced as the extraction temperature increased. For instance, at 120 ℃ the TP content ranged from 0.92±0.08 to 2.72±0.54 mg

GAE/g dry DSM. When the temperature was 150 ℃ the content ranged from 2.73±0.65 to 5.52±1.11 mg GAE/g dry DSM, and for 180 ℃ it was 6.31±1.4 to 12.13±1.1 mg GAE/g dry DSM. As shown in Table 1, the TP content continuously increased with temperature.

In the HT extraction without $CO_2$ addition, the maximum TP (12.13±1.1 mg GAE/g dry DSM) was obtained at 180 ℃ after 60 min, but an increased extraction time from 60 to 120 min resulted in a lower TP value. The decrease in TP content during high-temperature extraction could be associated with the degradation of thermolabile bioactive components in the DSM or longer exposure time. An example is the hydrolysis of lignan components, such as sesaminol glycosides, to polar lignans (sesaminol), simple sugar molecules, or organic acids. At relatively low temperatures (<150 ℃), TP increased with extraction time, but after 60 min the increment was insignificant. However, in the case of 180 ℃ the TP content continually decreased beyond 60 min, which could be a result of component hydrolysis due to thermal degradation. The addition of $CO_2$ to the HT process slightly increased the TP content, and when it was performed at 180 ℃ and 10 MPa a TP content of 15.94±1.56 mg GAE/g dry DSM was achieved, which was higher than that of HT-180 performed without $CO_2$ under the same operating conditions (12.13±1.1 mg GAE/g dry DSM). The lower water to $CO_2$ ratio (2:1, w/w) showed slightly higher TP values of 15.94±1.56 mg GAE/g dry DSM) than those of the higher ratio (4:1, w/w), which gave TP values of 14.07±1.81 mg GAE/g dry DSM. From this, it can be understood that the TP content is not significantly affected by pH changes. However, the TP content in the conventional extractions ranged from 1.31±0.52 to 3.86±0.72 mg GAE/g dry DSM, which is comparable to that of the HT extraction at 120 ℃. The

conventional extraction with 75% MeOH that was performed for 24 h gave the highest TP of 2.81±0.46 mg GAE/g dry DSM. However, the TP value obtained from the conventional method with 75% EtOH (1.31±0.52 mg GAE/g dry DSM) was found to be lower than that obtained from sesame meal under similar conditions by Nadeem et al.[19] (1.72 mg of GAE/g of dry DSM). Generally, the TP contents obtained from $CO_2$-assisted HT extractions were about 5-fold greater than those of conventional extractions.

Similarly, the total flavonoid (TF) contents obtained from HT extractions were greater (Table 7) than those obtained from the conventional extractions. However, the incremental differences in TF content were not as large as those recorded in TP content. The maximum TF contents obtained from HT, HT-$CO_2$, and conventional extractions were 4.86±0.52 mg quercetin/g of dry DSM, 6.07±0.81 mg quercetin/g of dry DSM, and 3.02±0.12 mg quercetin/g of dry DSM, respectively. Overall, the recovery of TF content was lower than that of TP content, which could be due to the thermal instability of flavonoids being greater than that of phenolic compounds. Generally, phenolic compounds are reported to show better resistance when treated at high temperatures compared to flavonoids[84].

Summarized bioactivity and antioxidant capacity are presented (Figure 3-4 a-b) for comparing the different extraction techniques. In both cases, $CO_2$-assisted HT extractions showed better recovery of the bioactive components and antioxidant activities than those of the other methods.

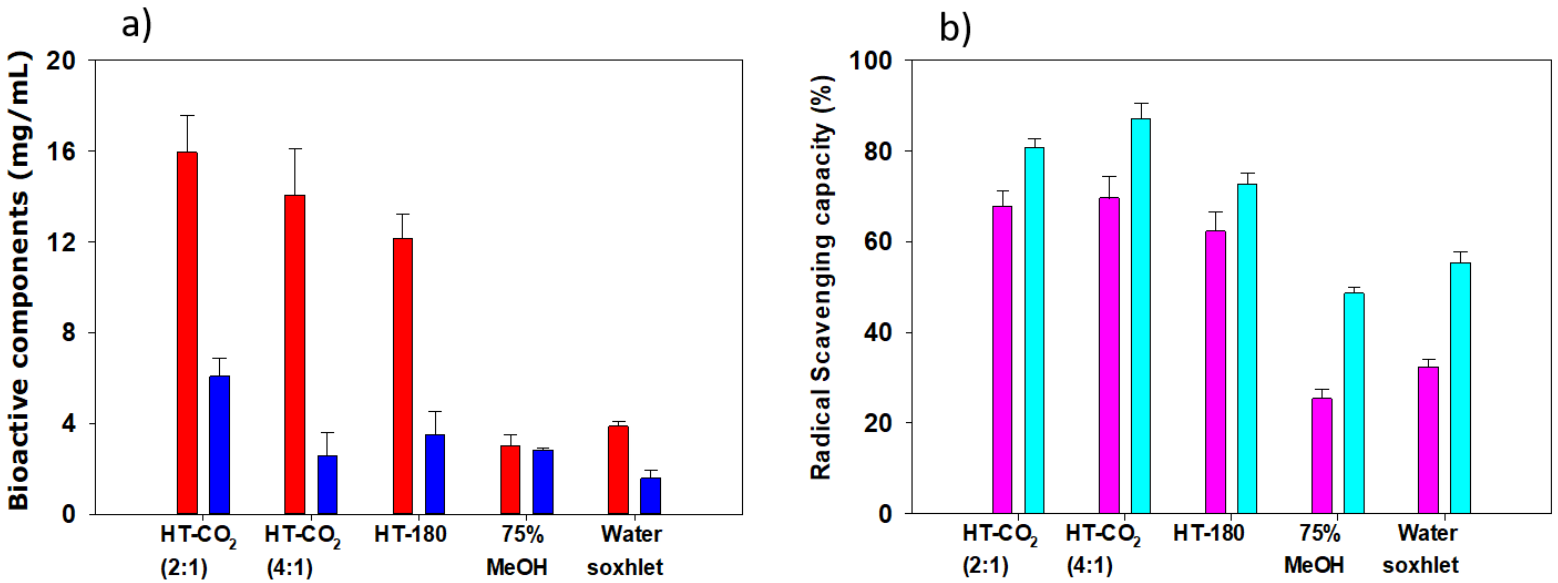



Figure 3- 4 a) Bioactive compounds. The red and blue bars represent the total phenol (TP) content (in mg GAE/ g dry DSM) and total flavonoid (TF) content (in mg quercetin/g dry DSM), respectively. b) Antioxidant activity. Purple and cyan bars represent DPPH and ABTs values, respectively, of: HT-180 (10 MPa, 60 min), HT-$CO_2$ (2:1, w/w; 180 ℃; 10 MPa; 60 min), HT-$CO_2$ (4:1, w/w; 180 ℃; 6 MPa; 60 min), 75% MeOH (100 ℃, 0.1 MPa, 24 h) and Water-Soxhlet (100 ℃, 0.1 MPa, 24 h).

The addition of $CO_2$ in the HT extractions enhanced the amount of bioactive compounds contained in the extract. When comparing the HT and HT-$CO_2$ extractions at 180 ℃ (Figure 3-4a), the TP content was seen to increase from the 12.13±1.1 mg GAE/g dry DSM obtained by HT to 15.94±1.56 mg GAE/g dry DSM obtained by HT-$CO_2$. However, the TF content showed only a slight increment when comparing the same extractions under the same conditions (Figure 3-4a). Generally, the TF content achieved by the HT extractions was comparable to those of the both conventional and water-Soxhlet extractions.

### 3.5.3. Antioxidant activity of DSM

The antioxidant activity of the DSM extracts was evaluated using DPPH and ABTS radical scavenging capacity assays based on Eq. (9) and Eq. (10) respectively. The extract antioxidant activity was more clearly presented in terms of $IC_{50}$ values, which were determined based on the DPPH radical scavenging capacities. The $IC_{50}$ value (which shows the amount of extract needed to reduce the number of free radicals by half) was calculated for each sample. Lower $IC_{50}$ values imply better radical scavenging capacity, and higher values imply poor antioxidant capacity. Based on the $IC_{50}$ values, extracts with lower $IC_{50}$ values from each extraction condition were selected for the analysis and as depicted in Figure 3-5 a and b, intense temperature conditions showed higher antioxidant activities in terms of DPPH and ABTS assays.

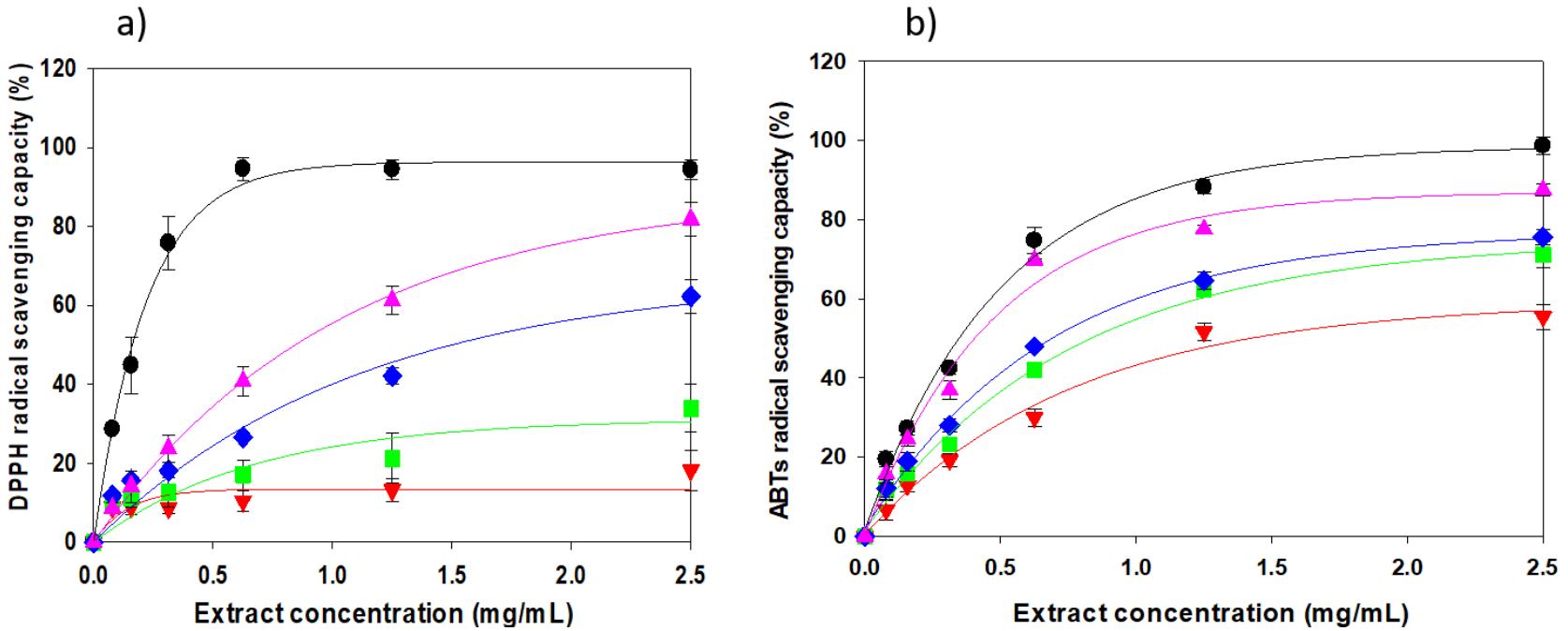



Figure 3- 5 a) DPPH radical scavenging activity of DSM extracts and b) ABTS radical scavenging activity of DSM extracts for HT-120 (down red triangles), HT-150 (green squares), HT-180 (blue diamonds), HT:$CO_2$ (4:1, w/w; purple triangles), and Trolox (control; black circles).

Accordingly, the highest DPPH radical scavenging capacity of 69.8% was obtained by an HT-$CO_2$ extraction at 180 ℃ after 60 min (Figure 3-5a). In general, the HT extractions performed at higher temperatures showed higher radical scavenging capacities than those performed at lower temperatures. The addition of $CO_2$ at 180 ℃ further increased the DPPH scavenging capacity from 58.78±3.2 to 69.83±5.1%. Based on the $IC_{50}$ values, conventional extractions showed better antioxidant activity than that of HT extractions at lower temperatures (Table 7). The extraction parameter effect was also observed in the extract colour and pH measurements of the hydrolyzates. The hydrolyzate colour changed from whitish to dark brown as the severity continued to rise.

Similarly, the $ABTS^+$ scavenging activity was determined to verify the results obtained from the DPPH radical scavenging assay. $ABTS^+$ radicals were effectively scavenged by the extracts obtained from HT extractions, but this was not evident in the conventional extracts. In both DPPH and ABTS assays, the scavenging activity increased linearly with an increase in extraction temperature.

The $ABTS^+$ scavenging capacity (Figure 3-5b) showed a gradual increase with temperature for the HT extractions. In the $CO_2$-assisted HT extraction at 180 ℃ (4:1, w/w), a higher scavenging capacity of 87.50±1.46% was obtained, which is higher than that of the HT extraction without $CO_2$ at 180 ℃ (75.57±0.9%). In general, both DPPH and ABT radicals were more effectively scavenged by HT extractions than they were by conventional extractions.

### 3.5.4. Phenolic content correlation with antioxidant activity

The antioxidant activity of the extract was mainly affected by the presence of bioactive compounds in the extract. Previous studies have shown that phenolic compounds have linear correlations with antioxidant activities[29]. As shown in Figure 3-6 a-c, the TP content showed a correlation with DPPH radical scavenging ($R^2$=0.9601, P<0.05) and the $IC_{50}$ values ($R^2$=0.9437, P<0.05), but a slightly lower correlation was obtained with $ABTS^+$ radical scavenging ($R^2$=0.8206, P<0.05). The correlation graph also clearly depicts the increase in antioxidant activity with increasing extraction temperature. The addition of $CO_2$ also caused an increase in the antioxidant activity. As shown in Fig. 5, the higher water to $CO_2$ ratio (4:1) showed better antioxidant activity than that of the lower ratio (2:1).

### 3.5.5. Severity effect on DSM

Since both temperature and extraction time are the key factors in determining the product of HT processes, the severity factor ($\mathrm{Log}\, R_o$), which shows the combined effect of temperature and extraction time, was used to evaluate the extraction yield and bioactive compound content for the HT extractions. The severity factor was calculated using the following equation (Equation 11):

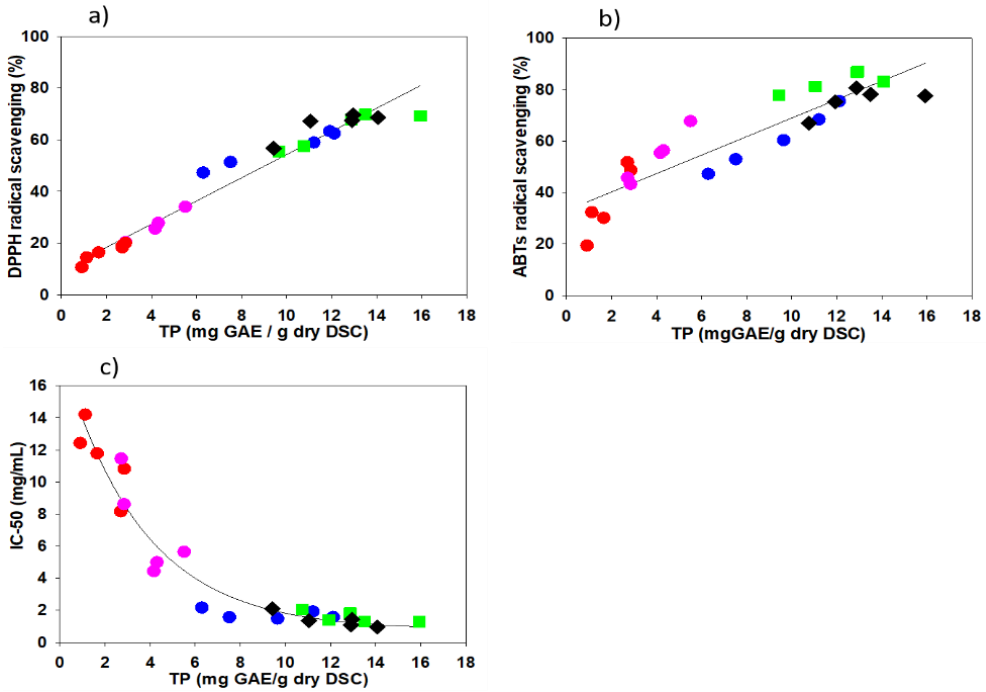



Figure 3- 6 Correlation of the TP content of the DSM extracts obtained using HT extraction at different extraction temperatures (120–180 ℃) and times (20–120 min) with their respective antioxidant activities. (a) DPPH˙ radical scavenging inhibition, ($R^2$=0.9601, P<0.05); (b) ABTS˙+ radical scavenging capacity, ($R^2$=0.8206, P<0.05); and (c) $IC_{50}$ value, ($R^2$=0.9437, P<0.05) for HT-120 (red circles), HT-150 (purple circles), HT-180 (blue circles), HT-$CO_2$ (2:1) at 180 ℃ (black squares), and HT-$CO_2$ (4:1) at 180 ℃ (green squares). Solid lines are the fitting curves of the experimental data.

$$\text{Log } R_o = \text{Log}\left[t * e^{\frac{T-100}{14.75}}\right] \quad (11)$$

Here, t is the extraction time (min), T is the operating temperature (°C), and 100 is the reference temperature (°C)[85]. The $\text{Log } R_o$ showed a better curve fit with extraction yield ($R^2$=0.93, P<0.05), and TP content ($R^2$=0.89, P<0.05), but exhibited a poor curve fit with TF content ($R^2$=0.55, P<0.05). All curves except that for the TF value from HT-$CO_2$ (2:1, w/w) fit well when $CO_2$ was added (Fig. 5b). The extraction yield was significantly increased from 7.1% to about 48.2% at $\text{Log } R_o$ values of 1.89 and 4.43, respectively (Figure 3-7). The TP content showed only a slight increase in the lower $\text{Log } R_o$ ranges (1.88 to 3.07). The maximum TP value of 12.13 mg GAE/g dry DSM was attained at a $\text{Log } R_o$value of 4.13, but gradually declined beyond this. This could be due to the prolonged exposure to intense temperature, which could severely affect the bioactive components in the extract. The bioactive compound content and extraction yield were slightly enhanced for the HT-$CO_2$ extractions. The maximum extraction yield obtained was 60.2% at $\text{Log } R_o$=4.43 during the HT-$CO_2$ (2:1) extraction, and the maximum TP value of 15.94 mg GAE/g dry DSM was obtained at $\text{Log } R_o$=4.13. The increase in extraction yield and TP content could be due to partially dissociated $CO_2$ producing a weak acid (carbonic acid), which might have also facilitated the hydrolysis of different components within the extracts, such as sesaminol triglycoside, sesaminol diglycoside, and polysaccharides.

However, in both cases (HT and HT-$CO_2$ extractions), the TF content did not show significant changes with increasing $\text{Log } R_o$. This shows that $\text{Log } R_o$ has less of an effect on the recovery of flavonoids than it does on the phenol content. The lower

TF content could also be a result of thermal degradation, as bioactive compounds are severely affected when exposed to high temperatures for a prolonged period of time. Generally, for HT extractions without $CO_2$ addition, the TP content showed only a slight increase in the lower $\text{Log}\, R_o$ ranges. As was mentioned, the maximum TP value for HT extractions was attained at a $\text{Log}\, R_o$ value of 4.13, but beyond this is started to decline.

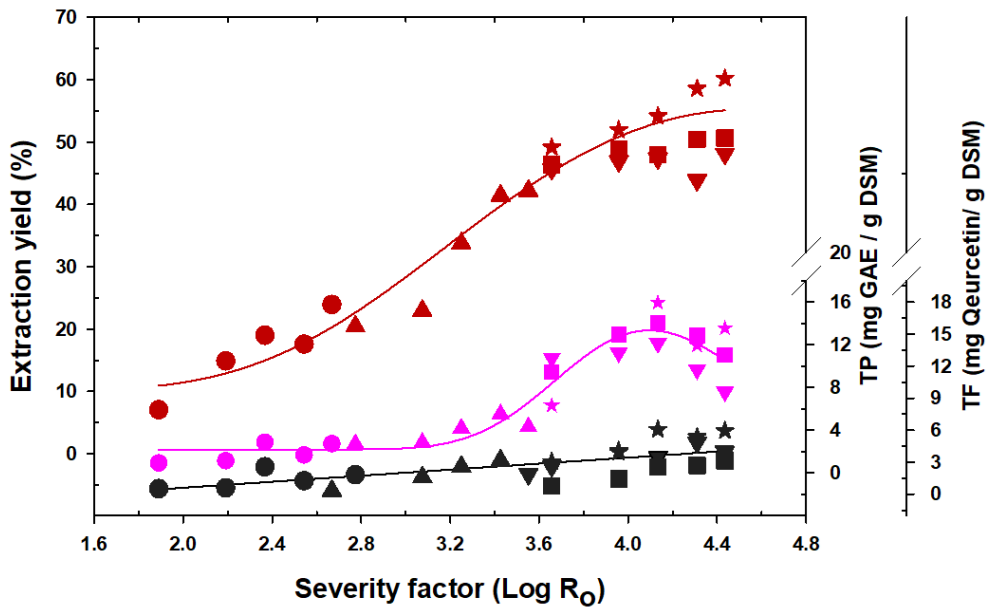



Figure 3- 7 Severity (Log $R_o$) effects on the extraction yield (%), TP content and TF content. Extraction yields for HT extractions at 120 ℃ (red circles), 150 ℃ (red triangles) and 180 ℃ (down red triangles). Extraction yield for HT-$CO_2$ (2:1) at 180 ℃ (red stars) and HT-$CO_2$ (4:1) at 180 ℃ (red squares). TP values from HT extractions at 120 ℃ (purple circles), 150 ℃ (purple triangles), and 180 ℃ (down purple triangles). TP values for HT-$CO_2$ (2:1) at 180 ℃ (purple stars), and for HT-$CO_2$ (4:1) at 180 ℃ (purple squares). TF values for HT extractions at 120 ℃ (black circles), 150 ℃ (black triangles), and 180 ℃ (down black triangles). TF values for HT-$CO_2$ (2:1) at 180 ℃ (black stars), and HT-$CO_2$ (4:1) at 180 ℃ (black squares).

However, for the HT extraction with $CO_2$ addition, the maximum TP value of 15.9 mg GAE/ g dry DSM was attained at $\text{Log}\, R_o$=4.13, with almost no significant changes on TF.

### 3.5.6. Extract identification and quantification

GC-MS analysis was performed to identify and confirm the bioactive compounds in the extracts through matching their mass spectra with those of the known compounds in the Mass Hunter GC-MS library. Some components that have previously been identified as bioactive plant extracts with antioxidant and inflammatory properties, such as pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3[86], were detected in the HT extracts. In addition, fatty acids (malic acid, oleic acid, and fumaric acid), sugar molecules (sucrose; maltose; α-D-mannopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-D-glucopyranose; and glucose-6-phosphate), amino acids (glycine, aziridine, L-valine), and other secondary metabolites were detected.

The majority of the identified chemicals were found in the extracts obtained from the HT extractions (Figure 3-8), while some chemicals, such as amidine and elaidic acid, were not detected in the conventional extractions. Some of the major compounds were D-lactose (RT=18.49, area percentage=0.97%), maltose (RT=18.42, area percentage=0.78%), galactopyranose, 1,2,3,4,6-penta (RT=18.30, area percentage=0.66%), α-D-xylofuranoside (RT=5.27, area percentage=0.341%), and glutamic acid (RT=4.49, area percentage=0.306%). On the other hand, most of the sugar molecules, such as maltose, sucrose, and raffinose, were detected in the conventional extractions. Generally, the GC-MS spectra confirmed the presence of major peaks in the extracts from the HT extractions, most of which were sugars and organic acids.

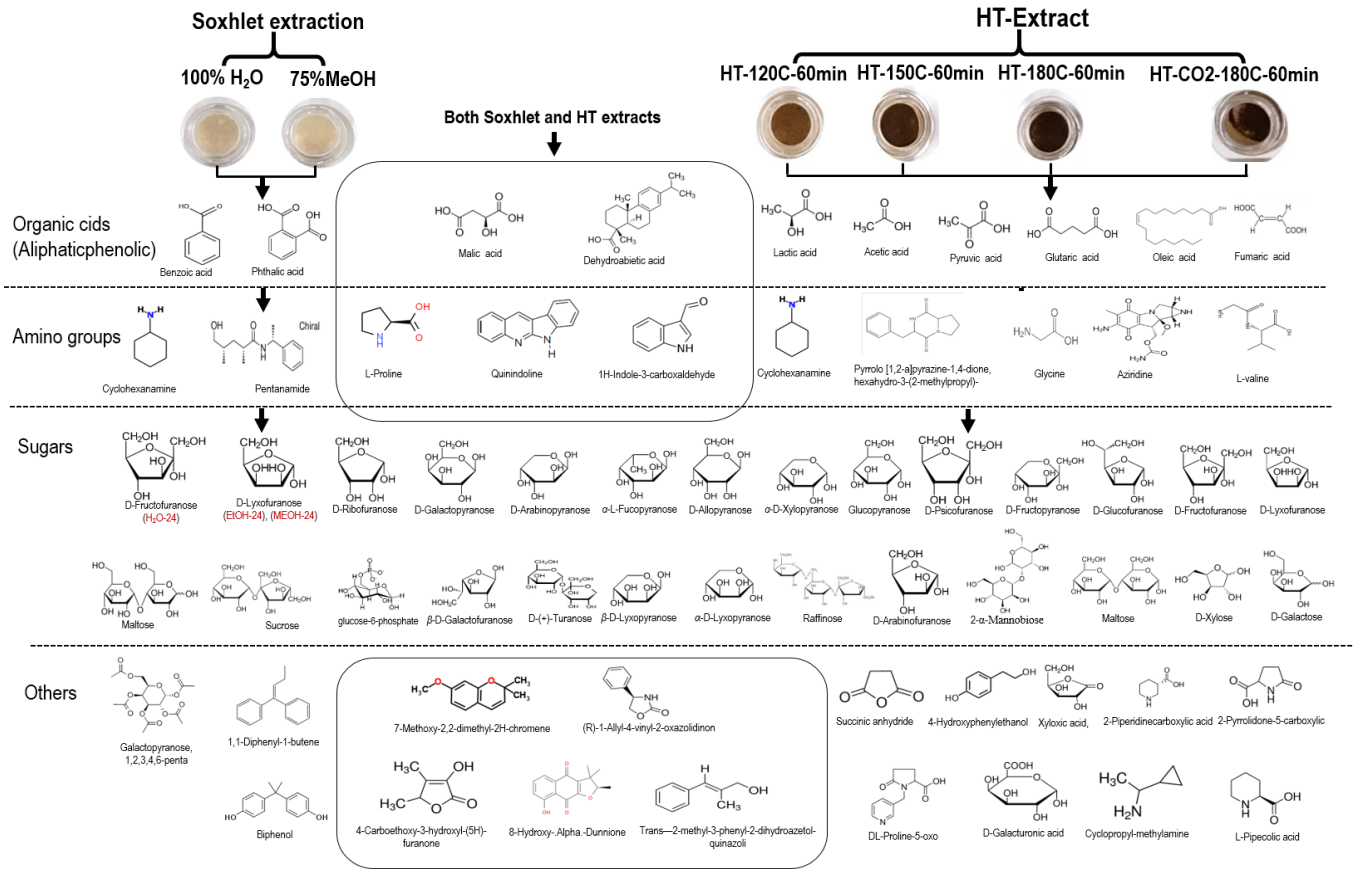



Figure 3- 8 DSM extract components analysed using GC-MS

### 3.5.7. Anticancer effect

The anticancer effects of DSM extracts were evaluated in MDA-MB-231 cells, and the experiments were performed in vitro. Previous studies have reported that the anticancer effects of DSM extracts mainly arise from the presence of water-soluble lignan components. Therefore, based on the $IC_{50}$ values we obtained (section 3.3), a total of three samples were selected (one from each technique) for the anticancer test. The extraction methods and their conditions for the three samples are as follows: HT extraction (180 ℃, 60 min), HT-$CO_2$ ($H_2O$:$CO_2$=4:1, w/w; 180 ℃; 60 min), and the conventional extraction (75% MeOH, 24 h). According to the results obtained in this study, all the extracts showed a cytotoxic effect on the cancer cells, but the cytotoxicity level varied with extract dosage. As illustrated in Figure 3-9, higher dosages showed a better cytotoxic effect on the cancer cells. However, as the extract concentration was increased, the cytotoxicity effect on normal cells (HDF) also became more severe (Figure 3-9b). From the figure, it can be seen that at an extract concentration of 2.5 mg/mL, the cancer cell viability for MDA-MB-231 decreased to 43.12±13.99%, 57.7±13.47%, and 75.16±7.06% when treated in the HT-180, HT-$CO_2$, and 75% MeOH extracts, respectively. However, the extracts showed cytotoxic effects (which are not desirable) on normal cells (HDF) at higher dosages (Figure 3-9b). Hence, to acquire the minimum side effect on normal cells, an extract dosage of 2.5 mg/mL was found to be the optimal concentration for cancer cell treatment with less cytotoxic effect on the normal cells.

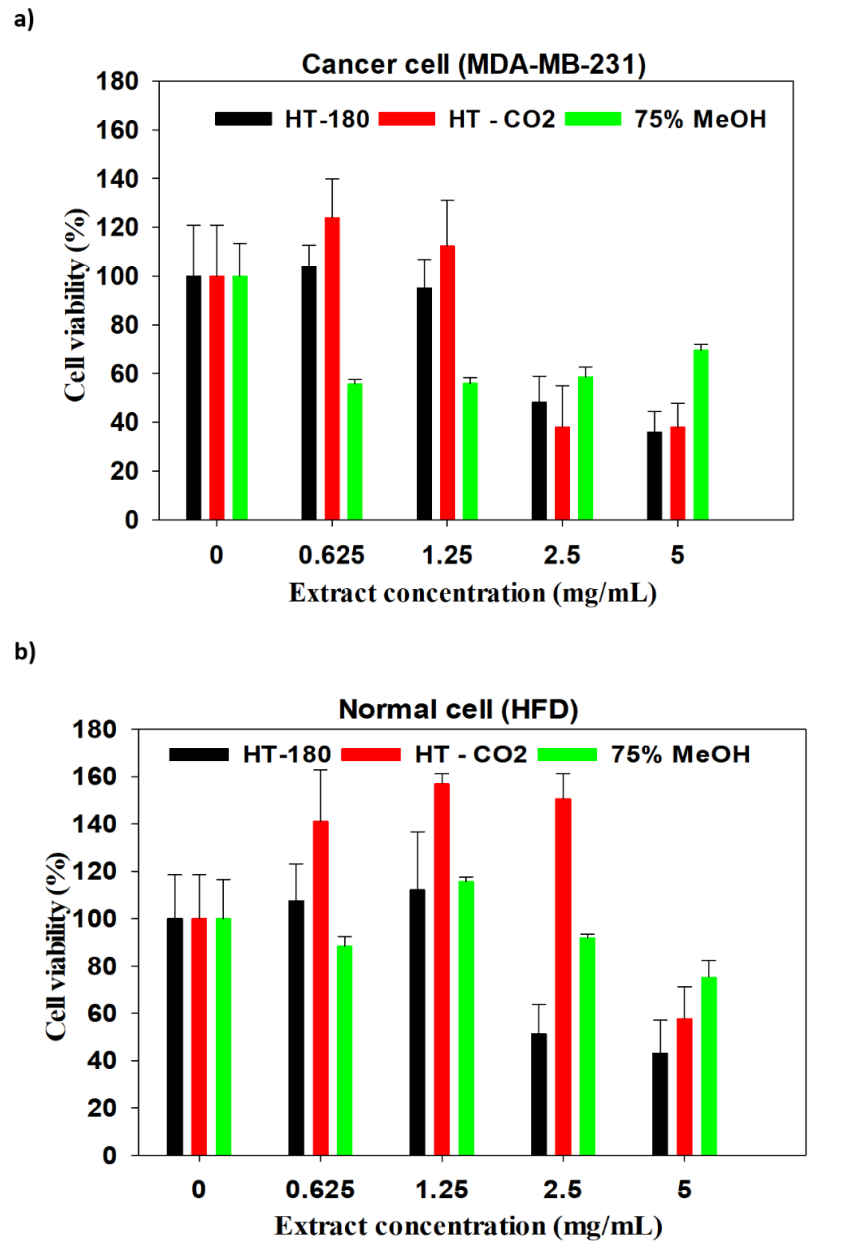



Figure 3- 9 Anticancer effect of DSM extract on (a) cancer cell (MDA-MB-231) and (b) normal cell (HDF) treated at different extract dosages for a period of 24h. HT-extraction performed at (180$^{o}$C, 60 minutes), HT-$CO_2$ ($H_2O$ : $CO_2$ at 4:1, w/w ratio) performed at 180$^{o}$C, 60min and 75% methanol at 24h

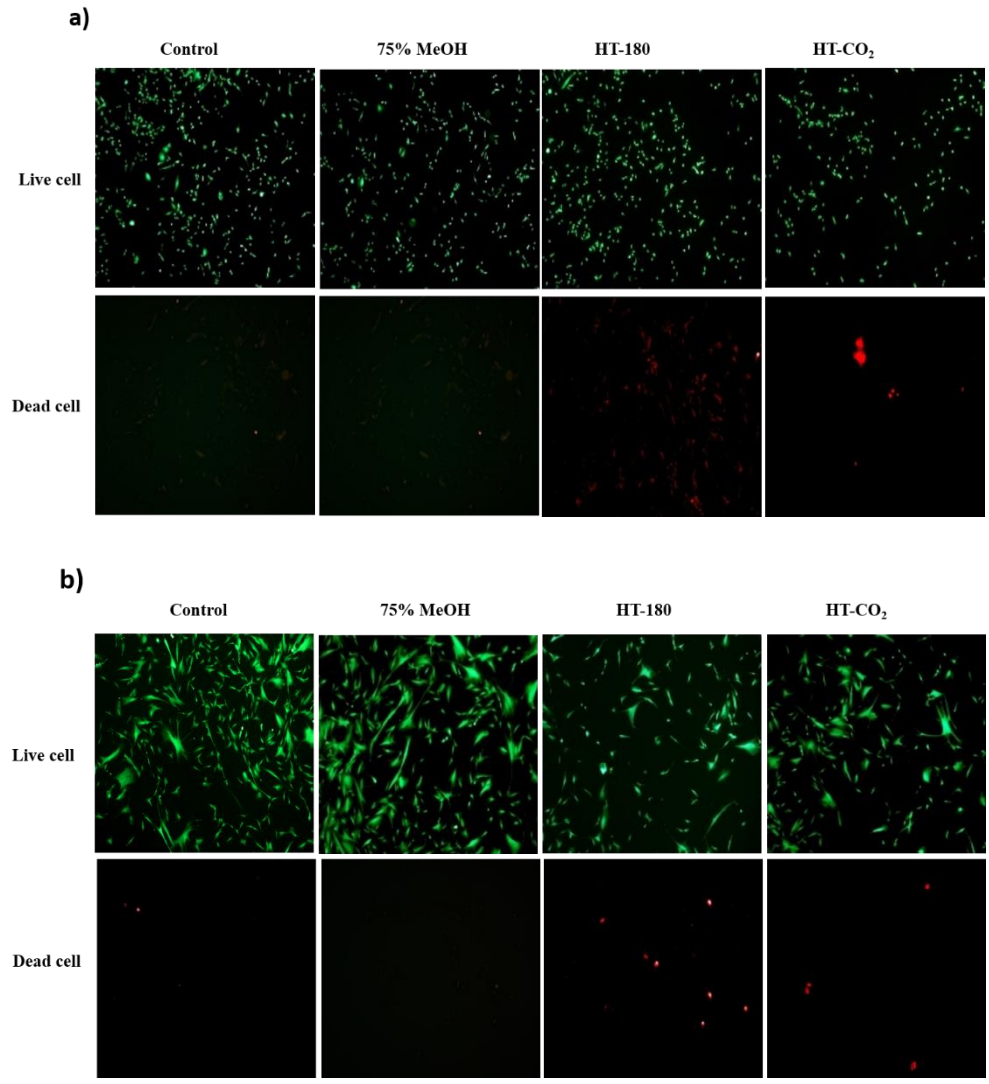



Figure 3- 10 Fluorescence microscopy images of (a) cancer cells and (b) normal cells at extract concentrations of 2.5mg/mL. green color indicates the presence of live cells and red color is an indication of dead cells in both normal and cancer cells. HT-extraction performed at (180$^{o}$C, 60 minutes), HT-$CO_2$ ($H_2O$ : $CO_2$ at 4:1, w/w ratio) performed at 180$^{o}$C, 60min and 75% methanol at 24h

The cytotoxicity effect with the 2.5 mg/mL dosage was then analysed using visualized fluorescence spectroscopy, where the live/dead cell imaging for the cancer cells (Figure 3-10a) and the normal cells (Figure 3-10b) was collected. Live cells for both MDA-MB-231 cells and the normal (HDF) cells were homogeneously stained green and dead cells were observed as red dots. Comparing the distribution of cell numbers, the HT-$CO_2$ extract showed a significant cytotoxic effect on the MDA-MB-231 cells and a lesser anti-proliferative effect on the normal cells.

# Chapter 4. Conclusion

The acidic transformation of sesamolin to sesamol under supercritical carbon dioxide using solid catalyst (Amberlyst-15) was efficiently performed. The maximum sesamol yield of 88.07% and maximum conversion of sesamolin of 98.1% was recorded at 120$^{o}$C and 120 minute of reaction time. Compared to the previous techniques used, here there are many advantages achieved in this method. There first is that higher percentage conversions are recorded. Second, this process completely replaces toluene (toxic solvent) with an environmental friendly solvent ($CO_2$). Third, in this research work lignan transformation was achieved without the addition of strong oxidizing agents such as hydrogen peroxide. Fourth, this process avoids the need for downstream processing, such as product purification since $CO_2$ is easily separated by depressurization.

The other important work in this research is, the recovery of water soluble bioactive components from the solid residue (defatted sesame meal) using $CO_2$ assisted hydrothermal (HT) process. HT extraction was successfully applied to recover bioactive compounds from DSM, and the addition of $CO_2$ increased this recovery and enhanced the antioxidant activity of the obtained compounds. While conventional extractions use organic solvents, these HT extractions used green solvents (water and $CO_2$). It also required shorter extraction times than those of conventional methods, increased the amount of collected bioactive components, and enhanced the antioxidant effects of those components. In the $CO_2$-assisted HT extractions, temperature and extraction time were manipulated to effectively recover greater amounts of the bioactive components. Furthermore, the HT-$CO_2$ extract with an $H_2O$:$CO_2$ ratio of 4:1 (w/w) showed higher antioxidant activity than the

conventional technique. The DSM extracts of the HT, HT-$CO_2$, and conventional (75% MeOH) extractions all showed cytotoxic effects on cancer cells while having little effect on normal cells.

## 4.1 Future work

In this research work, the lignan transformation was performed in a batch reactor, mainly due to process simplicity, and the requirement of relatively small amount of reactants and catalyst. However, in the case of mass production of upgraded sesame oil (sesame oil rich in sesamol) a continuous process is inevitable. In fact, the lignan transformation can easily be integrated into an existing Sc-$CO_2$ sesame oil extraction process, when the required reactor design parameters are performed. Additionally, integrating the two processes will save the amount of carbon dioxide needed, since the same carbon dioxide introduced for extraction is also used during the lignan transformation. As shown in figure 4-1, after sesame oil extraction the product is directed to a reactor (packed catalytic reactor) in which the lignan transformation takes place. The catalyst (Amberlyst-15) has a strong acidic nature due to the sulfonic functional group embedded on the surface of solid matrix. Hence, compressed $CO_2$ can easily get into the inner catalyst matrix (styrene polymer) in which case it makes it to swell and expose the inner active sites of the catalyst ensuring rapid mass transfer between the catalyst and supercritical reaction phase. Moreover, the lignan transformation can be achieved without the need for additional equipment such as pumps, since the extract can be directly introduced to the reactor due to the build-up pressure in the extractor. Sc-$CO_2$ oil extraction is often performed at pressures above 200 bar in order to get maximum yield. Consequently, lignan transformation can be effectively achieved at less than 180 bar according to the data

obtained in this research paper. The reactor pressure can also be controlled by a back pressure regulator, and the reaction temperature can be maintained by a jacketed heating system so that $CO_2$ will be at supercritical state at all times during the reaction. In the final product small amounts of unreacted water can be obtained due to a carry on with the discharged gaseous $CO_2$, and this water residue can be removed by heating. Along with changing the reaction system from batch to continuous, the process can also be scaled up, as continuous processes are often favourable for large scale productions. However, laboratory scale lignan transformation in a packed catalytic reaction should be performed in order to use an accurate parameters fitted from process data. Additionally, having the product and reactant concentrations of the reaction, it's much easier to investigate the reaction kinetics for the bioactive component recovery. The lignan transformation and DSM extraction were performed from 10 to 120 minutes, hence a reaction model can be developed to present the reaction trends and also to predict the product output beyond the reaction times through the use of a simulated reaction model. However, the mechanisms and pathways of lignan transformation have not been well elucidated and this could be a problematic in developing accurate model. Therefore, more investigation of the lignan transformation mechanism in Sc-$CO_2$ is required to better understand the reaction and this research work can be used as an input for further studying lignan transformations.

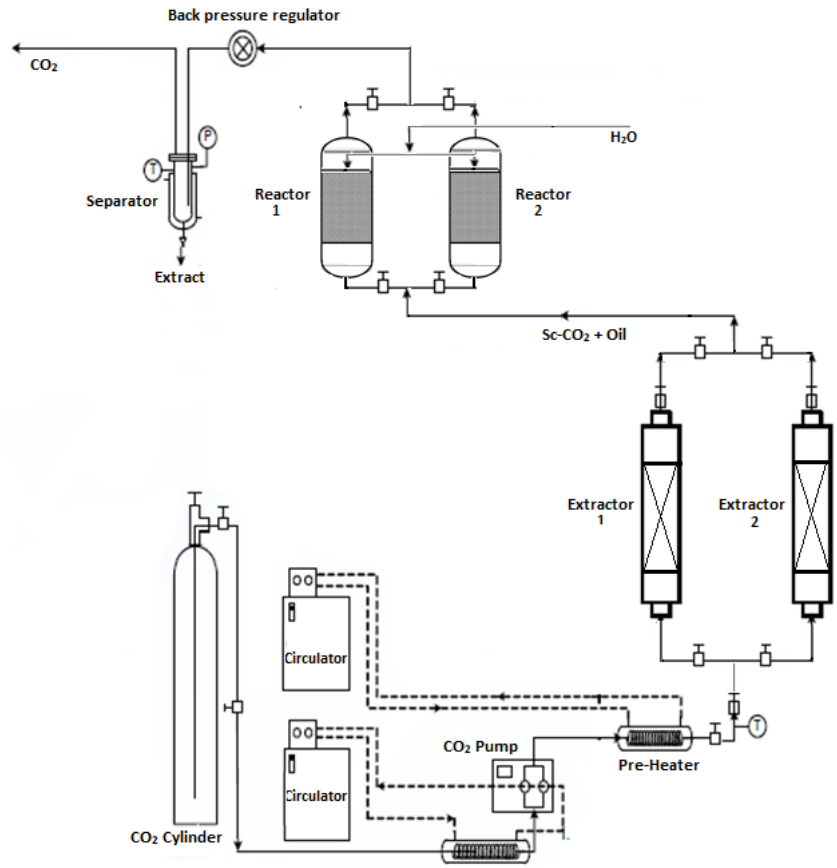



Figure 4- 1 Supercritical carbon dioxide sesame oil extraction and lignan transformation.

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## Abstract

# Sesame seed lignan transformation and recovery under supercritical carbon dioxide

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Sesame seed is one of the well-known oilseed with rich source of nutritive values. It is ranked 2nd next to flax seed, in its lignan content. It is considered to be one of the most valuable oilseed primarily because of the presence of highly bioactive lignan components. These components gave sesame oil excellent stability due to the strong antioxidant properties. Sesamin and sesamolin are the main lignans in sesame oil, and relatively very small amount of sesamol also exists. Actually, sesamol does not exist naturally in sesame seed, rather it's derived from sesamolin during sesame seed pre-treatment such as roasting, bleaching, and degumming. Even after the treatments only small amount of sesamol is detected in the oil. However small the amount of sesamol is, it exhibits the strongest antioxidant property compared to both sesamin and sesamolin.

For this reason, the acidic conversion of sesamolin to sesamol under supercritical carbon dioxide was performed using solid catalyst (Amberlyst-15) having strongly acidic functional group. Previously, the transformation of sesamolin to sesamol and

samin was performed in toluene, with the addition of strong oxidizing agents such as hydrogen peroxide. However, in this research work, sesamolin under supercritical $CO_2$ was efficiently transformed mainly to sesamol and small amount of other lignans such as samin and sesaminol. Here, no oxidizing agent was used, only small fraction of water was added to furnish hydroxide ion in the solution. The two main advantages of using supercritical $CO_2$ is; first it replaces relatively toxic chemical (toluene) with a green solvent ($CO_2$). Second, product purification is not important, as $CO_2$ can easily be separated by depressurization.

The other important work in this research is, the recovery of water soluble bioactive components from the solid residue (defatted sesame meal) using $CO_2$ assisted hydrothermal process. Defatted sesame meal (DSM) just like sesame oil, it is also rich source of pharmaceutical components. With the ever-growing demand for novel natural drugs, the utilization of green and efficient techniques to obtain highly biologically active natural products from unexplored herbal/medicinal plants is becoming increasingly important. Herein, the efficiency of green hydrothermal extraction (HT-extraction) for the recovery of bioactive compounds from DSM was investigated. The effects of the extraction temperature, extraction time, and ratio of $CO_2$ added on the product yields and physicochemical, and structural properties of the DSM extracts were systematically investigated. In addition, the antioxidant activities of the HT extracts were examined using different *in vitro* methods, such as $DPPH^{\bullet}$ scavenging capacity assay, and $ABTS^{\bullet+}$ scavenging capacity assay, and the correlations between the composition of the HT extract and the antioxidant activities of the resulting extracts were analyzed to determine the origin of the antioxidant

activity of the extracts. Both extraction temperature and time were found to influence the extraction yields and product quality.

The addition of $CO_2$ even at lower temperature has showed the presence of more bioactive compounds and better antioxidant activities were achieved compared to conventional extraction. In vitro, anticancer cell effects on human breast cancer cells were checked for the extract solutions and a promising anticancer effect with lesser effect on normal cells was observed.

**Keyword:** Sesame lignan, antioxidant, bioactivity, transformation, Supercritical carbon dioxide, hydrothermal process

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