KAUNAS UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMICAL TECHNOLOGY

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Fractionation of fenugreek (*Trigonella foenum-graecum L.*) seeds and analyses of their fractions composition and properties

Master's Degree Final Project

Supervisor
Assoc. prof. dr. Jonas Damašius

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KAUNAS, 2017

KAUNAS UNIVERSITY OF TECHNOLOGY

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Food Science and Safety (code 621E40001)

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DECLARATION OF ACADEMIC INTEGRITY

5 Jun 2017

Kaunas

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CONTENT

Hamidioglu, Irfan. *Fractionation of Fenugreek (Trigonella foenum-graecum L.) Seeds and Analyses of Their Fractions Composition and Properties*. Master's thesis in Food Science and Safety / supervisor assoc. prof. Jonas Damašius. The Faculty of Chemical Technology, Kaunas University of Technology.

Research area and field: Technological Sciences, Food Technology

Key words: *Trigonella foenum-graecum L, fermentation, fractionation, antioxidant activity, fatty acid composition.*

Kaunas, 2017. 90 p.

SUMMARY

Fenugreek (*Trigonella foenum-graecum L.*) is a natural herbal plant which has several health benefits, antioxidant activity, anti-inflammatory, anti-diabetic, anti-carcinogenic properties and consumed all around the world since the ancient times. It has been used in folk medicine to lowering level of cholesterol in the bloodstream and reducing diabetes. Fenugreek seed is a great source of protein, dietary fibre, vitamin, mineral, edible oil, and bioactive components and mostly used for food consumption. However, to separate components from the fenugreek seed might be more beneficial to use it for various purposes such as food supplement, the source of natural medicine, media for the microorganism growth, and use in animal diet.

Therefore, the aim of this work to obtain various fractions from the fenugreek seed to analyse their fractions and properties. Four major fractions were obtained respectively: fenugreek ethanol fraction (FEF), fenugreek oil fraction (FOF), fenugreek fermented fraction (FFF), and fenugreek powdered fraction (FPF). The main processes were applied in the following order in appropriate terms and conditions: extraction of fenugreek seed with ethanol, Supercritical Fluid Extraction-Carbon dioxide (SFE-CO_2) extraction to obtain oil, fermentation application on modified fraction using with *Lactobacillus casei* microorganisms and lyophilization (freeze drying) process to obtain powdered fractions. Chemical compositions, total phenolic content, antioxidant activity, fatty acid composition, GC-MS analysis and microorganism determination were done for the fractions with the appropriate method used. Several compounds which possess antioxidant properties were identified in FEF. An increase in the number of microorganisms was observed in FFF. It was investigated characteristic of fenugreek oil composition. In general, all fractions evaluated and discussed at different points of their properties.

Hamidioglu, Irfan. *Vaistinės Ožragės (Trigonella foenum-graecum L.) sėklų frakcionavimas ir jų frakcijų sudėties bei savybių analizavimas*. Magistro baigiamasis projektas / vadovas doc. dr. Jonas Damašius; Kauno technologijos universitetas, Cheminės technologijos fakultetas.

Mokslo kryptis ir sritis: Technologijos mokslai, Maisto technologijos

Reikšminiai žodžiai: *Trigonella foenum-graecum L, fermentacija, antioksidacinis aktyvumas, riebalų rūgščių kompozicija, bioaktyvūs junginiai*.

Kaunas, 2017. 90 p.

SANTRAUKA

Ožragė (*Trigonella foenum-graecum L.*) yra natūralus vaistinis augalas, kuris turi naudingų sveikatai savybių, antioksidacinį aktyvumą, priešuždegiminių, antidiabetinių, priešvėžinių savybių ir yra vartojama visame pasaulyje nuo senovės. Ji buvo naudojama tradicinėje medicinoje sumažinti cholesterolio kiekį kraujyje bei sumažinti riziką susirgt diabetu. Ožragės sėklos yra geras baltymų, ląstelienos, vitaminų, mineralų, aliejaus, bioaktyvių junginių šaltinis ir daugiausiai naudojama kaip maistas. Išfrakcionavus komponentus iš ožragės sėklų, jie gali būti naudojami kaip maisto papildai, kaip medikamentai, kaip terpė mikroorganizmams augti bei kaip kaip pašaras gyvūnams.

Šio darbo tikslas yra išgauti įvairias frakcijas iš ožragės sėklų ir išanalizuoti šių frakcijų savybes. Buvo gautos 4 pagrindinės frakcijos: etanolinė ožragės frakcija, aliejinė ožragės frakcija, fermentinė ožragės frakcija ir miltelinė ožragės frakija. Buvo taikomi keli metodai: sėklų ekstrakcija etanoliu, superkritinė skysčių ekstrakcija CO_2 dujomis (SKE-CO_2) išgauti aliejų, fermentacija naudojant *Lactobacillus casei* bakterijas ir liofilizacija (sausas džiovinimas) miltelinės frakcijos išgavimui. Buvo atlikta cheminės sudėties, bendro fenolinių junginių kiekio, antioksidacinio aktyvumo, riebalų rūgščių, GC-MS analizės ir mikroorganizmų nustatymas atskirose frakcijose. Etanolinė ožragės frakcija buvo nustatyta keletas junginių, kurie pasižymi antioksidaciniu aktyvymu. Taip fermentinėje frakcijoje buvo pastebėtas didesnis mikroorganizmų skaičius. Buvo nustatyta ožragės sėklų aliejaus sudėtis. Apibendrinant, buvo įvertinta ir aptarta visų frakcijų savybės.

LIST OF ABBREVIATIONS

FEF	Fenugreek ethanol fraction
FOF	Fenugreek oil fraction
FFF	Fenugreek fermented fraction
FPF	Fenugreek powdered fraction
LD50	Lethal Dose, 50%
GC/MS	Gas Chromatography- Mass Spectrometry
GRAS	Generally Recognized as Safe
HPLC	High performance Liquid Chromatograph
ESI	Electrospray ionization
ASE	Accelerated Solvent Extraction
PUFA	Polyunsaturated fatty acid
SC-CO2	Supercritical Fluid Extraction-Carbon dioxide
SSF	Solid state fermentation
LF	Liquid fermentation
DPPH	2, 2-diphenyl-1-picrylhydrazyl.
ABTS	2, 2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
LDL	Low-density lipoprotein
SDF	Soluble dietary fibre
MRS	Man, Rogosa and Sharpe

INTRODUCTION

Natural plants and spices have been used since earliest time, people have used herbal plants for various purposes. Some kind of natural plants could effect on human metabolism in a positive way. The use of several plants has spread to a variety of areas such as social, economic and religious. Throughout the history, especially with the development of the food technology, plants have been involved to be used in natural food additives, source of the natural aroma, medicine, and functional food application even in the perfumery industry. Many natural herbal plants have been cultivated for the economic benefits.

Fenugreek (*Trigonella foenum-graecum L.*) is a plant which belongs to family Fabaceae, and it has very important properties. Fenugreek leaves generally used as an herb and seed of fenugreek used as a spice. It is cultivated in many countries. The plant leaves and seeds is great source of protein (4-Hydroxyl-isoleucine, trigonelline**)**, vitamin (Vitamin C, Beta- Carotene, Choline), mineral (Calcium, Magnesium, Potassium, Sodium, Iron, Sulphur), fat (unsaturated acids, polyunsaturated fatty acids (PUFA), Linoleic, Oleic acids , Linoleic acids), carbohydrates (soluble dietary fibres) and bioactive compounds (Galactomannan, Diosgenin, 4-Hydroxyl-isoleucine, Carpaine, Arginine, Coumarin, Caeffic acids, Scopoletin, Chlorogenic acids, Scopoletin, Hymercromone, p-coumaric acids). Also, fenugreek leaves and seed has been reputed for their several pharmacological effects including gastro protective, antipyretic, hypercholesterolemia, antibacterial, antioxidant, appetite stimulation, hypoglycaemia, and anti-inflammatory. The presence of several bioactive compounds in fenugreek might be support for its health benefits.

In Turkey, fenugreek being used commercially and has great economic value. Commonly used as spicy, flavouring agent on culinary, and traditionally used for pastırma (dried meat) production to protect the meat from a harmful microorganism. Therefore, in this study aimed to obtain various fractions from fenugreek seed and analyses of their properties. On the other hand to separate different fraction from the fenugreek and to apply on it various purposes such as fermentation, food supplement, source of natural medicine, and animal feed due to the reason that it could get more benefits from the plant material, to get economically advantage, and might be promising for the furthers studies. Some of the possible ways to obtain different components in plant material might be productivity, effective, and with the high yield of the process such a supercritical CO_2 extraction method to obtain oil from the seed. Freeze drying process might be effective to obtain commercially valuable fractions from the liquid form which has many disadvantages for the storage and shelf life. Some of studies have been completed previously with the fenugreek seed and it has been applied to a bakery, meat industry to

improve the quality of the products and enriched their antioxidant, antimicrobial activity. Moreover useful microorganisms such as *Lactobacillus* species could be successfully fermented in a medium combined with the fenugreek seed and could be used on foods as a new probiotic product or supplemented on the foods.

Aim and tasks of the work

The main aim of the work was to obtain various fractions of fenugreek seed and analyse of their properties. The following tasks were raised to achieve the aim of the study:

1. To analyse the chemical composition of fenugreek seed;
2. To obtain fractions of fenugreek seeds: ethanol fraction (FEF), oil fraction (FOF), fermented fraction (FFF) and powdered fraction (FPF);
3. To analyse chemical composition of FEF and determine the antioxidant properties;
4. To evaluate fatty acid composition of FOF and characterise the oxidation properties;
5. To estimate microbiological properties and volatile compounds of FFF;
6. To analyse chemical composition of FPF.

Structure and content of the thesis

The thesis is written in English. It consist of the list of abbreviations, summary, introduction, literature survey, materials and methods, result and discussion, conclusions, references, Curriculum Vitae (CV), dedication and acknowledgements. The thesis has 90 pages, 24 tables, and 19 figures. The list of references includes 159 sources.

1. LITERATURE SURVEY

1.1. General characterization of fenugreek (*Trigonella foenum-graecum L.*)

Fenugreek (*Trigonella foenum-graecum L.*) is a plant consumed all around the world since ancient times for different purposes such as spicy, green vegetable, making food and medicinal purpose. The plant is indigenous to western Asia and south-eastern Europa. The name of the species means Greek hay (*Foenum-graecum*) [1]. The plant is semi-arid crop and belongs to the family Fabaceae. Generally cultivated in Turkey, India, Morocco, Israel, Tunisia, United States, Canada, Spain, Russia, North Africa and the Mediterranean countries. Moderate climate, low rainfall, and regular temperatures are good for cultivation of fenugreek plant [2]. Fenugreek is a very important crop for commercially and it has been an important plant species for mankind with many features that it possesses. The plant and seeds are reputed for their special aroma and bitter taste [3].

Fig. 1.1. Botanic view of fenugreek plant

Fenugreek should be cultivated straight into a well-drained soil sunlit spot. Usually planted time between April and August. Germination takes place in a week and plant grows quickly. Fenugreek is the leguminous plant that could harbour nitrogen-binding bacteria in roots, therefore it has the capability to fix atmospheric nitrogen in the land. The plant is a crop that does not need much water, with the arid environment and limited water source can reduce the cost of an irrigation system, besides it prevents contamination of groundwater sources. Fenugreek is a strong plant against most weeds and it fairly resists against some frost during the cultivation. When fenugreek seed germinated, it composes a seedling, finally germinated seeds develops into stems, pods, flowers, and seed. After the

swollen seed, the rootlet emerges from the seed coat, pass through from seed coat and initiates primary roots growth [4; 7; 11].

Morphological characteristic of fenugreek described and shown in Table 1.2 [1; 4; 55]. Small leafed variety could grow slowly during the winter period but the larger variety generally dies off. Fenugreek sometimes does not fix nitrogen, therefore plant should be carefully planted at the appropriate site. Indigent soil can affect flavour and nutrient of the crop. Tolerated pH range 5 to 8 for the optimum growth of a plant. Crop becomes ready to harvest between 4-5 months [3; 6]. The plant has grown a wide range of climatic condition and different countries, that shown in Table 1.1. All over the world, approximately 500 commercial products which contain fenugreek have been reported by natural medicines database [6]. The plant and seed have great economic value. In addition, a plant is commercially used for cosmetic products.

Fenugreek is widely cultivated in Turkey and traditionally used for pastırma (dried meat) production. The sauce known in Turkey by the name of "Cemen" is consist of crushed fenugreek seed, garlic, chili pepper, a mixture of several spices and water. The paste of cemen is in use of covering the slice of pastırma, it gives flavour and protects the meat from spoiling [5]. Most of the times fenugreek have been reported as a natural medicine because of its positive health benefits. In India fenugreek has been reported of the Indian diet in excess of thousands of years and in Egypt, a plant has been planted since thousands of year BC. According to some reports, India is the native home of fenugreek. Egyptian people used fenugreek leaves as a constituent of holly smoke in fumigation. Fenugreek was used in ancient Rome to initiate childbirth birth and used after birth [7].

Table 1.1. Different countries that grow Fenugreek

Continent	**Country**
Asia	India, China, Israel, Iran, Lebanon, Japan, and Pakistan
Europe	Greece, Portugal, Russia, Spain, Switzerland, Turkey, UK, France, Germany, Austria
Africa	Sudan, Tanzania, Morocco, Kenya, Egypt, Ethiopia, Tunisia
America	Canada, United States, Argentina

Fenugreek seed and leaves have been used as a spice to enhance the taste of vegetable and meat dishes in Africa, south of Asia and Mediterranean area. Fresh leaves are consuming as a green vegetable in some countries [2]. Leaves and seeds are useful for some chronic disease as a cough, swellings, and diabetes. There are several health benefits of fenugreek seed reported [7]. Seed is rich in vitamins, fresh fenugreek leaves the good source of protein and has beneficial on several treatments. Besides, fenugreek is rich in phytochemicals. Different components have various activities and

important components as trigonelline control blood sugar in diabetic patients. Clinical works indicated that, the statistically important decline in human serum LDL cholesterol, triglycerides, and total cholesterol by fenugreek consumption [8]. In the pharmaceutical industry, sapogenin which presents high amount in fenugreek seed, used on birth control pills. That could be commercial interest for growing fenugreek. Seeds used in some countries as feed for lactating animals and to increase cow milk [9].

Table 1.2. Morphological characteristics of fenugreek (*Trigonella foenum-graecum L.*)

Morphological Char.	**Description**	**Dimensions**	**Ref.**
Stem	Greeny, slightly quadrangular, generally characterized pinkish because of presence of anthocyanin under field	0.5-1 cm / diameter	[1; 4]
Leaf	Trifoliate, clearly petiolate, leaf lamina oval, the leaf lamina and petioles changes from greenish to pinkish	1.5-4.5 / 0.8-1.5 cm	[1; 4]
Seed	Modify in color form brownish to golden yellow, Rectangular to oval in shape, deep grooves between the radicle and cotyledon.	10-20/pod, 3-5 mm / 2-3 mm	[4; 55]
Flower	At the beginning yellow, white on maturity	1.6 - 2.2 cm	[4]
Petiole	Hairy, usually anthocyanin tinged, pale green	0.5-1.1 mm	[4]
Wings	Papery, White	4.5 - 5.5 mm	[1]
Stigma	Pale green, glaucous	1.5 - 2.1 mm	[4]
Plant habit	Straight or plenty branched, Steep or weak	20-130 cm in length	[55]

Fenugreek has been privileged “Generally Recognized as Safe” (GRAS) status by the United States Food and Drug Administration whereas allergic findings for some patients [10]. Components which exist in fenugreek seed such as galactomannan, diosgenin, steroids, which have possible to influence on cholesterol and glucose levels in humans, it could exposure risk to health problems however remarkable clinically detrimental adverse effect because of consumption fenugreek as food supplement have not been reported [8]. Despite that, it has been reported that several patients had an allergic reaction as abnormal skin reaction, bronchospasms, wheezing and diarrhoea against the consumption of fenugreek.

Some side effect problem has been reported for consuming fenugreek seed. Furthermore, possible chemical compounds of fenugreek interact with different drugs pending patient medication. Several negative side effect has been reported for children and pregnant woman by consuming fenugreek in an oral way [11].

Table 1.3. Common names of fenugreek

Languages	**Name**
Latin	Trigonella foenum-graecum L
Turkish	Çemen otu
Lithuanian	Vaistinė Ožragė
Indian	Methi
Arabic	Hulba
French	Bockshorklee
Spanish	Alholva
Italian	Fieno greco

Taxonomy of Fenugreek

Fenugreek (*Trigonella foenum-graecum* L.) is belonging to the family of Fabaceae, a genus of the plant is Trigonella. The subfamily belongs to Papilionaceae. Different type of species has different chromosome number. Trigonella foenum-graecum has belonged to a commonly grown species. In some reports, fenugreek is classified into four groups according to with information of pigment in the seed coat, seed weight and seed fluorescence action under Ultraviolet (UV) light. [12].

Table 1.4. Classification of fenugreek

Name	**Classification**
Family	Fabaceae (Leguminosae)
Subfamily	Faboideae (Papilionaceae)
Subtribe	Trigonellinae
Order	Fables (Leguminales)
Species	*Trigonella foenum-graecum*
Tribe	Trifolieae
Genus	*Trigonella*
Kingdom	Plantae

Classification of fenugreek is shown in Table 1.4 [4], and fenugreek called in different language shown in Table 1.3. Important fenugreek names are *Trigonella foenum-graecum L*. in Latin, Senegre in French, Çemen otu in Turkish, Vaistinė Ožragė in Lithuanian, Methi in Indian, Koroba in Japanese, Tipilina in Greek, Hulba in Arabic and Alholya in Spanish. According to the various reports, from 1920 to present time many scientists differently categorized to fenugreek and Dangi *et al*. (2004) [13] proposed on fenugreek species that originated in Turkey [13].

1.2. Chemical composition and properties of fenugreek (*Trigonella foenum-graecum L.*)

Fenugreek plant has various chemical components. Seeds and leaves have many beneficial compounds. Proximate chemical composition of fenugreek seeds shown in Table 1.6 [4; 16; 41; 56; 57] and chemical components of fresh fenugreek leaves shown in Table 1.5 [4; 16; 56; 57]. Seed is a source of protein, vitamin, carbohydrate, mineral, bioactive compounds, dietary fibre, volatile compounds, anti-oxidants, nutraceutical compounds steroids and etc. Leaves are rich in mineral and vitamin. Seed contains nearly 20- 30 % protein, 5-8 % fat, 5-11 % moisture, 45-55 % fibre, and 6 % starch. Chemical composition depends on different ecological condition [16].

Table 1.5. Chemical composition of fresh fenugreek leaves

Name of component	Fresh fenugreek leaves
Protein	4.4 %
Fat	1.0 %
Fibre	1.0 %
Moisture	86 %
Calcium	395 mg/ 100g
Magnesium	67 mg/ 100g
Potassium	31 mg/ 100g
Sodium	76 mg/ 100g
Vitamin C	52 mg/ 100g
Choline	1.35 g/ 100g
Sulphur	167 mg/ 100g

Fenugreek seed has a bitter taste because of the presence of saponins. The oil from an extraction of fenugreek seeds has essentially neutral lipids, approximately 10 % phospholipids, and 5.4 % glycolipids [15]. Characteristic of seed oil similar like commonly edible oils. Fresh fenugreek leaves contain approximately 4-5 % protein, around 1-1.5 % fat and fibre, and big amount (80-85 %) consist of a moisture. According to the different reports, the moisture content of fresh fenugreek leaves and seed based on differences time of harvesting, storage conditions, and the varied environmental conditions [6]. Ash content of dry fenugreek seeds reported around 3-4 % and ash content changes due to the reason of different soil conditions [16]. The protein content of seeds could be attributed to different environmental conditions. Fenugreek has many biological, pharmacological, nutraceutical and medicinal properties because of having the chemical constituents such as polyphenolic, volatile compounds, amino acids, alkaloids, steroids and bioactive compounds [17].

Table 1.6. Chemical composition of fenugreek seed

Name of component	Fenugreek Seed
Protein	28.55 %
Lipids	6-7 %
Total fibre	48.0 %
Ash	3.2 %
Carbohydrates	46.25 %
Moisture	5.22 %
Starch	6.0 %
Gum	20.0 %
Total ethanol soluble sugars	8.06 %
Calcium	160 mg/ 100 g
Magnesium	160 mg/ 100 g
Potassium	530 mg/ 100 g
Sodium	19 mg/ 100 g
Iron	14 mg/ 100 g
Vitamin C	50 mg/ 100 g
Beta- Carotene	96 μg/ 100 g
Choline	50 mg/ 100 g
Trigonelline	380 mg/ 100 g

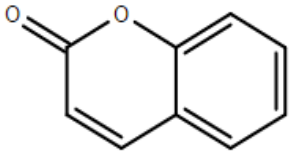
Bioactive compounds are very important for human body system. Various important beneficial compounds of fenugreek plant shown in Table 1.7 [1; 4; 62; 63]. Several scientific studies have been reported that major bioactive compounds of fenugreek are; 4-hydroxyisoleucine, galactomannan, diosgenin, quercetin and trigonelline. Fenugreek is rich in phenolic compounds. Phenolic compounds have potential health benefits primarily because of their antioxidant properties. There is a relationship between consumption of food which rich in phenolic compounds affect the low rate of coronary heart disease, particular forms of cancer and stroke [18]. Clinical studies have been reported that fenugreek has properties such anti-inflammatory, antitumor, antiviral and hypotensive effect. Fenugreek seeds contain diosgenin that has potential preventing agent against colon cancer [8]. Steroidal sapogenins are given the capability of producing soap-like foaming characteristic. Polyphenols have anti-oxidative properties. Various class of flavonoids and isoflavanoids occurs on fenugreek [4]. Scientists have identified around 150 volatile compounds while investigated on fenugreek. Volatile compounds of fenugreek are providing flavour and aroma to the fenugreek seed. The main volatile compounds are; anethol, camphor, fennel, sesquiterpene hydrocarbons, furan and heterocyclic compounds. These

compounds are also responsible for fenugreek flavour. Volatile substances give similar flavour like curry and maple syrup [19]. Fenugreek seeds contain a higher amount of mineral comparing to the other kind of seeds. Pyrazines is the compound that gives toasted flavour into fenugreek seed. Some aromatic compounds of fenugreek; sesquiterpene, *n*-alkanes, hexanol, and γ-nonalactone. Fenugreek contains lecithin, mucilage and approximately 75% of digestible dry matter [15].

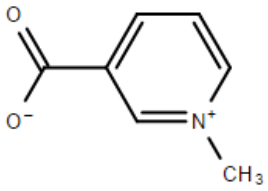
Table 1.7. Important biological compounds present in fenugreek

Group of Compounds	**Selected Compounds**	**Ref.**
Protein / N-compound	4-Hydroxyl-isoleucine, trigonelline.	[1], [4]
Lipids	Neutral lipids, phospholipids, glycolipids	[63]
Phenolic compounds	Coumarin, caeffic acids, scopoletin, chlorogenic acids, scopoletin, hymercromone, p-coumaric acids.	[62]
Flavonoids	Orientin, kaempferol, vitexin-7-O-glucoside, quercetin, isoquercitrin, vitexin, isovitexin.	[1]
Aroma compounds	Pyrazines/ sotolone	[4]
Sapogenins / Sapogenin- peptide ester	Diosgenin, fenugreekine ,Graecunin B, C, D, E and G	[62]
Antioxidants and phenols	Orthodihydroxy phenols, coumarines, total flavonoids.	[63]
Main bioactive compounds	Galactomannan, diosgenin, 4-Hydroxyl-isoleucine, carpaine, arginine, coumarins	[1], [4]
Isoflavonoid phytoalexins	Medicarpin, maackiaian, vestitol, and sativan	[4]

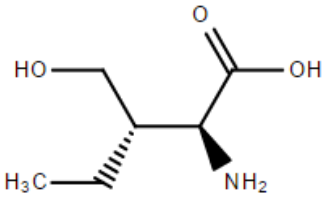
Common biologically active compounds present in fenugreek seed



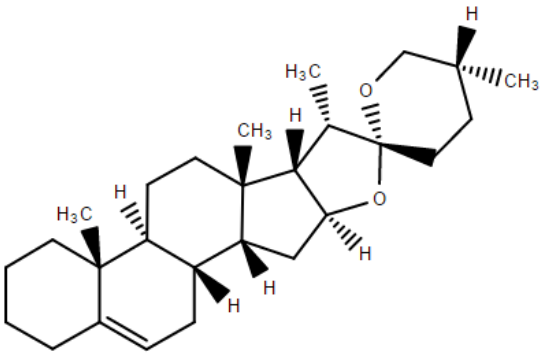
Coumarin / $C_9H_6O_2$



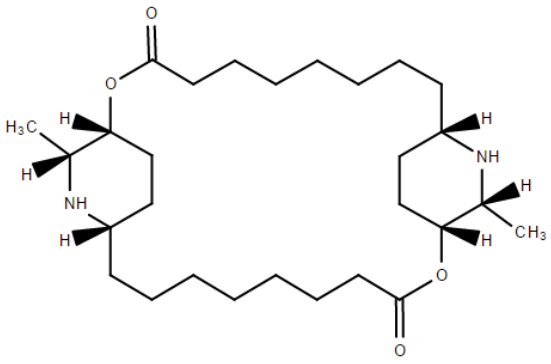
Trigonelline/ $C_7H_7NO_2$



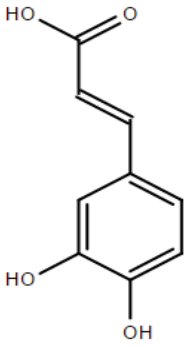
4-Hydroxyl-isoleucine / $C_6H_{13}NO_3$



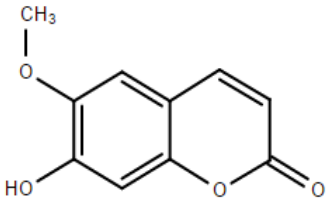
Diosgenin / $C_{27}H_{22}O_3$



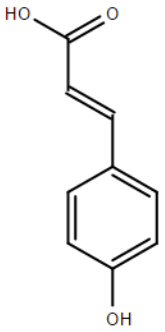
Carpaine (alkaloid) / $C_{28}H_{50}N_2O_4$



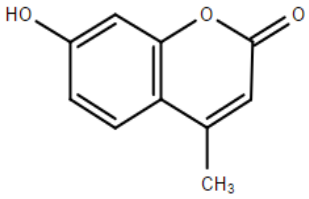
Caeffic acid / $C_9H_8O_4$



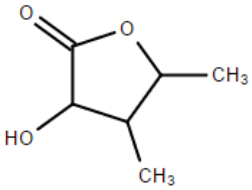
Scopoletin / $C_{10}H_8O_4$



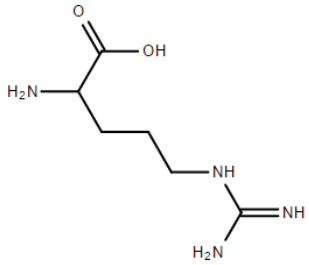
p-coumaric acids / $C_9H_8O_3$



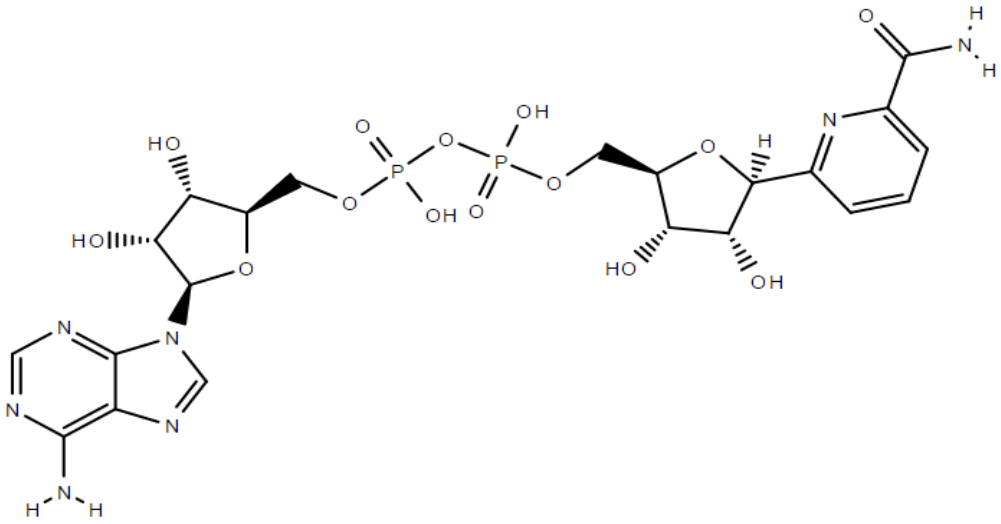
Hymercromone / $C_{10}H_8O_3$



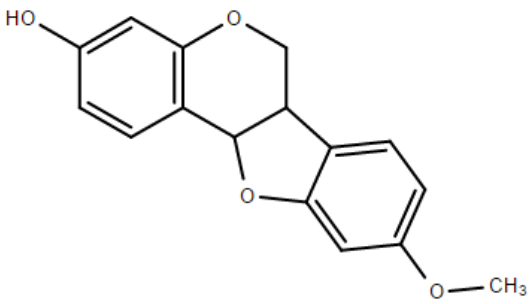
Sotolone
(aroma compound) / $C_6H_8O_3$



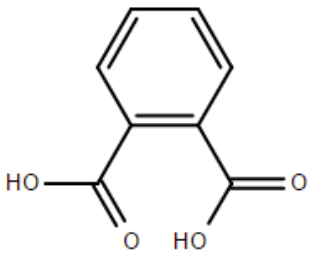
Arginine / $C_6H_{14}N_4O_2$



Fenugreekine/ $C_{21}H_{27}N_7O_{14}P_2$ / Sapogenin peptide ester

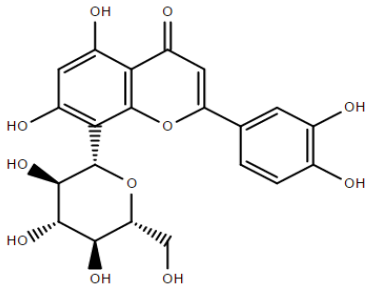


Medicarpin/ $C_{16}H_{14}O_4$
(Isoflavanoid)

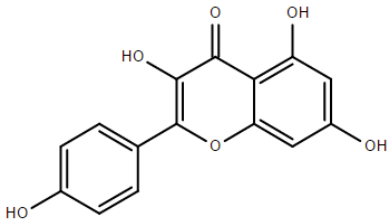


Phthalic acid / $C_6H_4(COOH)_2$

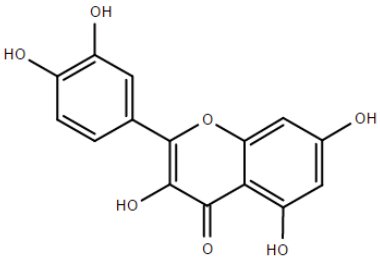
Common flavonoid derivatives present in fenugreek



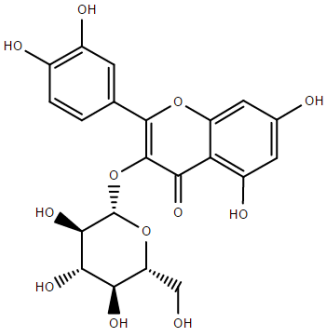
Orientin / C21H20O11



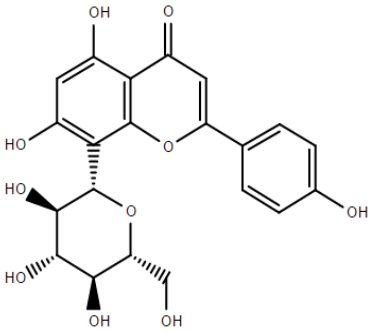
Kaempferol / $C_{15}H_{10}O_6$



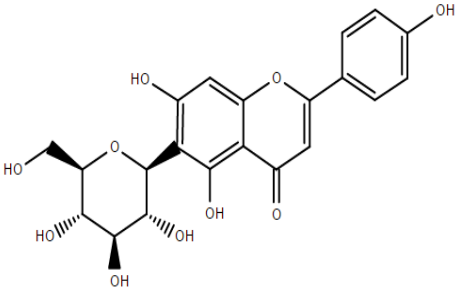
Quercetin / $C_{15}H_{10}O_7$



Isoquercitrin / $C_{21}H_{20}O_{12}$



Vitexin / $C_{21}H_{20}O_{10}$



Isovitexin/ $C_{21}H_{20}O_{10}$

1.2.1. Leaves

Fenugreek leaves contain vitamin, mineral, saponins and usually strongly fragrant. It is petiolate, pinnately trifoliolate and leaflets are toothed. Leaves nerves usually get over into teeth and inflorescences short. The plant growth straight. It is rarely branching. Leaflets are oblong, 10-35 mm long, 5-15 mm wide, the shape of leaves are almost equal, 1-5 cm long and haired. The whole plant and leaves possess anthocyanin [20]. It used as greens and vegetable, it has pungent taste and leaves are having a smooth surface. For crop, harvesting time is around 4-5 months and while ripening or maturity period leaves become yellowish after a start to fall. It is very important timely harvesting. Early harvest, grains stay immature. While late harvest, seed loss from pod because of bursting [4].

Fig. 1.2. Botanic view of fenugreek leaves

1.2.2. Seed and pods

Pods of fenugreek plant are long, it has sickle shape and pointed. A colour of pods turns from green to the brown or yellowish brown while growing. As the physical appearance of pods is rarely straight, curved and 10-20 cm in length, 0.2-0.6 cm in width. Each of pods is included around 8-20 seeds. Fenugreek seeds form is changing rectangular to round form. Seeds colour depends on type and variety [21]. A major specific colour of fenugreek seeds is brown, light green and cinnamon colour. Fenugreek plant has single or double podded. The plants which contain a high level of bioactive compounds have a double pod.

Fenugreek seeds are very hard, not easy to grind and large seed variety is plenty around Mediterranean region, small seed varieties dominate eastward. Species are wild in the Canary Islands, South and North Africa, Australia and European countries. Average length from 4.01 to 4.20 mm, width range from 2.35 to 2.62 mm, thickness range between 1.50-1.75 mm and geometric mean diameter from 2.40 to 2.67 mm [21]. The seed of fenugreek has a large amount of galactomannan gum, therefore seed extract used in the food industry as food emulsifier or thickening agent. Furthermore, seeds are a good source of food colouring dye [11].

Fenugreek seed increases the appetite, eliminate bad odour from mouth. Main storage organ in the seed is testa (seed coat). The coat surrounds to seed and separated from the embryo. The weight of the seeds according to the Slinkard *et al*. (2006) [22], averages around 15-20 gram / thousand seeds. Some case, extraction of fenugreek seeds used in flavour imitation. Vanilla, maple syrup, rum and butterscotch flavours are the main flavouring used for imitation [22].

Fig. 1.3. Botanic view of fenugreek seed

Galactomannan presence in the seed is important for storage. The long chain of polysaccharide provides a longer term of storages and changes cell wall thickening of cells. Galactomannan is also supporting the growth of seedling. Fenugreek seeds have a bitter taste. The seed can be eaten cooked or raw, and the seed protein and fibre has no taste and aroma. Bitterness aroma comes from seeds oil

and mainly compounds are steroidal saponins and alkaloids. The seeds of fenugreek contain a small amount of starch however compared with another legume, the seed contain a higher ratio of minerals and 0.02-0.05 % of seed oil is consist of volatile compounds [23]. Fenugreek seed contains various compounds such a flavonoids, coumarins, carotenoids, and another compound with very less LD50 values [24].

Commercially seed oil and extract could be used because of its valuable compounds. For the future studies, fenugreek seeds and leaves are very promising natural plant material. It gives great opportunities to improve future trend application on functional foods. Especially galactomannan, diosgenin, several bioactive compounds which fenugreek has, could be attractive to investigate and improve on the food.

Secondary metabolites of fenugreek

Secondary metabolites are necessary for the plant to survive in their environment and metabolism of plant produces a huge amount of unique compounds. Primary metabolism of plants provides genetic information of plants to the proteins, carbohydrates and amino acids, thus all simple physiological processes which necessary to the growth of plant provided by primary metabolism. For the plant's ideal growth and development, there is a great balance between primary and secondary plants products, including the ability to adapt to changing environmental conditions [25]. Secondary metabolites of plants give plants characteristics such as physical appearance, colour, and some defensive properties.

Plant secondary metabolites divided three major group based on their biosynthetic origins. These groups are respectively; flavonoids and polyphenolic compounds, Nitrogen-containing alkaloids and sulphur-containing compounds, terpenoids. In some cases, secondary metabolites of plants attracted commercial interest, because of their properties. In the food industry, essential oils, alkaloids, and glycosides from plant materials are used as flavouring agents and food colorants. Especially secondary metabolites are a great source for pharmaceutical industry owing to various properties [26]. Fenugreek seed has important secondary metabolites. At the present time, new methods are investigating for isolation and classification of fenugreek seed steroids. Immobilized cells and plant cell suspension cultures have been used for producing important phytochemicals from plant materials. Scientists have been completed several studies on isolation and biosynthesis of galactomannan and diosgenin from fenugreek seed. Several techniques have been applied to tissue culture, cell suspension culture, and biological manipulations to increase the secondary metabolites

of fenugreek seed [27]. For further research, it is possible to make more efficient ways to isolate and investigate of various important compounds of fenugreek.

1.3. Fractionation and extraction of different compounds from fenugreek seed

Extractions of plant origin material from natural herbs and seeds are the old process used by humankind. In old times, herbs were extracted in daily activities as preparation of tea and used in health care. With the progress of the science, simple extraction process turned into various fractionation and separation process. Different and new techniques contributed to the development of the food science and phytochemistry. Generally, the first step of the chemical analysis is extraction [28]. The various method used for separation compounds from the mixture of liquids or solid bodies by a suitable solvent. Good extraction process should carry the compounds which we want to extract into solution cause less change on the structure of compounds and easy for furthermore analysis.

Extracts obtained from plants are comparatively impure semisolids, liquids or powders and may be ready to use as improvement agent or fractionated into divided individual chemical compounds [29]. The extract of plants is providing unrestricted opportunities for the biotechnological area because of unique availability of chemical diversity.

According to the several reports, the phytochemicals from plants generally safe and extensively alternatives with few adverse effect. It possesses beneficial biological activity. In most cases, different fractions and extracts from plants claimed good benefits for health. Fractionation is a separation process that used to divide phase transition into smaller quantities. Fractionation process is also used in the food industry as fractioned milk and produced a different viscosity of various oils from seeds [30]. Several methods exist for isolation of natural substances from plants. Most common methods are extraction using with organic solvent and distillation with water. Ethanol, methanol, hexane, acetone and methylene chloride are an organic solvent that uses for the extraction process. Different organic solvent has different properties and extracted substances are concentrated by removing the solvent by methods as distillation. However organic solvents are mostly toxic and concentration of solvent should be finely reduced in the last products.

Because of the fact that plant extracts possess different various phytochemicals and their different polarities give complication for separation, characterization, and identification of them. For identification of herbal extracts, there are several methods are used as high-performance liquid chromatography, thin-layer chromatography, and column chromatography [31].

Fenugreek seeds are a natural source of beneficial compounds. For this reason, it has attracted significant interest for scientific studies. Different fractions and extractions of fenugreek seeds have been studied last three decades. In addition, there are commercial uses for the different fractions of the seed. In the food industry, seeds fractions and extracts are used as an ingredient for imitation flavours [7]. Fenugreek oleoresins are similar like maple flavours. Seed flavourings ingredients which semi-solid extracts composed and obtained by evaporation have effective on black walnut, butter, nuts, and butterscotch.

Diosgenin is used by pharmaceutical industry and currently is using in the drug industry for steroid hormones production. Saponins have antioxidant, foaming, antimicrobial, flavouring and anticarcinogenic properties. [8]. Many chemical compounds of fenugreek seeds could be used as emulsifier, antioxidant, surfactant, ingredients for functional food and cosmetic industry. In developing countries, fenugreek seeds easily attract the interest of scientists with its effective components. Various fraction and extraction of fenugreek seeds are inspired by new studies for future.

Previously methods like; HPLC/ ESI/ DAD and ASE used for the purpose that analyses fenugreek seeds extracts. Mainly used organic solvents are; methanol, ethanol, dichloromethane, acetone, hexane and ethyl acetate for a various extract of fenugreek seed. Also, hot/cold water could be used for extraction.

Several studies have been completed with different extraction process of fenugreek seeds. Bioactive components of fenugreek seed has been extracted with ethanol and evaluated by using MS/ GC [33]. Various important bioactive compounds and their properties are shown in table 1.8 [33], from the report fenugreek contains phytochemical such Aziridine, 1, 2, 3- trimethyl-, trans-, 2-Propen-1-amine, Nethyl-, 3-O-Methyl-d-glucose and Dibutyl phthalate [33]. Those compounds possess such as antimicrobial, anti-cancer, anti-inflammatory, hypocholesteroemic properties [8]. Pasricha (2014) [61] investigated on dried sample of fenugreek seed, and leaves. It was used water and methanol for the extraction**.** It was identified several phytochemicals which are very important for the body functional system. Some of the compounds which was found; Propanoic acid, 2-methyl-, 9,12-Octadecadienoic acid (Z,Z)-, methyl ester, 9,12,15-Octadecatrienoic acid, ethyl ester, n-Hexadecanoic acid, 3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, 2-Pentadecanone, 6,10,14-trimethyl, 2-Pentadecanone, 6,10,14-trimethyl, and Vitamin E. The presence of those compounds indicate that the fenugreek seed and leaves has useful compounds for treating and inhibit several diseases such as heart diseases, diabetes and lung cancers [8].

Table 1.8. Several chemical compounds from ethanolic fenugreek seed extract obtained by GC/MS.

Compound name	**Mol. Form.**	**Activity**	**Nature of Comp.**
Aziridine, 1,2,3- trimethyl-, trans-	C5H11N	Antimicrobial	Nitrogen compound
1- Azabicyclo [2.2.2]octane, 4-methyl-	C8H15N	Antimicrobial	Nitrogen compound
2-Propen-1-amine, Nethyl-	C5H11N	Antimicrobial	Nitrogen compound
á-D-Glucopyranoside, methyl	C7H14O6	Preservative	Sugar moiety
Hexane, 3-bromo-	C6H13Br	Antimicrobial	Bromo compound
Heptanoic acid, 2-ethyl-	C9H18O2	-	Fatty acid compound
Dibutyl phthalate	C16H22O4	Antimicrobial	Plasticizer compound
3-O-Methyl-d-glucose	C7H14O6	Antifouling	Sugar moiety
1-Dodecyne	C12H22	-	Alkene compound
Bicyclo[3.1.1]heptan-3- one, 2,6,6-trimethyl-	C10H16O	-	Ketone compound
Piperidine, 1,1'- methylenebis-	C11H22N2	Antimicrobial Anti- cancer A. inflammatory	Alkaloid
1-Octanol, 2-nitro-	C8H17NO3	Antimicrobial	Nitrogen compound
Squalene	C30H50	Antioxidant Antimicrobial Pesticide Anti-cancer	Triterpene
9,12-Octadecadienoic acid (Z,Z), phenylmethyl ester	C25H38O2	Hypocholesteroemic	Fatty acid ester

1.3.1. Water and ethanol seed extract

Ethanol is the organic solvent which used for extraction of plant material. It is preferable solvent because of its properties. Ethanol solvent could be categorized "bio-solvent". Ethanol obtained by the fermentation process and used sugar-rich crops as cereals and sugar beet. It is flammable and explosive. Ethanol is high in purity, low polarity has a low price and biodegradable. According to the features of ethanol, it is used on a large scale. Because of the toxicity of the methanol and another organic solvent which has a toxic effect, ethanol could be an effective solvent for using extraction of fresh plant and seeds [32]. Usually, fenugreek seeds were extracted with methanol, acetone, hexane, ethyl acetate, dichloromethane because of different solvents have different polarity and they have different nature to extract compounds from the herbs and seeds. Therefore water could be used as a solvent to isolate polar compounds such as sugars, polysaccharides, amino acids, proteins, and enzymes. Most of the herbal extracts from plants and seeds which prepared traditionally are aqueous

extracts. Several studies have shown that fenugreek seeds extract from ethanol and water have wide range biological effect and properties as antioxidant and anti-microbial [34].

Several studies have been completed with fenugreek seed. Fenugreek seed was extracted used with different solvents or water, and different parameters have been applied such as the temperature of media, concentration of extracts, and different amount of bioactive, phenolic and flavonoid components has been found in a different amount [35]. According to the Norziah *et al.* (2015) [35] crude fenugreek seed extracted with ethanol, methanol, and water, as a result, shown in Table 1. 9. Therefore, water extracted fenugreek seed (germinated) showed highest antioxidant activities.

*GAE: Gallic acid equivalents; CE: catechin equivalents. ± Standard deviation (n= 3), a–e Values with various letters in the same column are significantly different ($P < 0.05$).

Table 1. 9. Phenolic and flavonoid amount of fenugreek seed extracts.

Crude Extracts	Total flavonoid (mg GAE/ g)	Total phenolic(mg CE/ g)
Extract from seed powder		
Water extract	3,76 +/- 0,4[a]	19,31+/- 0,2[a]
Methanol extract	9,48 +/- 0,1[c]	43,15+/- 3,6[c]
Ethanol extract	14,2 +/- 0,5[d]	44,96 +/- 2,8[c]
Hot water extract	7,30 +/- 0,4[b]	25,60+/- 0,2[b]
Extract from germinated seed		
Water extract	38,5+/- 0,9[e]	156,3 +/- 2,8[d]

1.3.2. Oil from seed

Fenugreek oil has a yellowish colour, bitter taste, fetid odour and obtained from fenugreek seeds. Studies have shown that oil range from fenugreek seeds between 5-8 % and composition of oil nutritious and it has beneficial properties. Mainly fenugreek oil is consisting unsaturated acids, polyunsaturated fatty acids (PUFA) and volatile compounds. Because of its health benefits and unique properties seed oil is very useful [36]. Scientific reports proved that essential oils from the edible plant as fenugreek with high polyunsaturated fatty acids are recommended on diets for lowering blood cholesterol [37]. Linoleic, oleic and linoleic acids are main compounds found in fenugreek seed oil. Like many other seeds oils, fenugreek seed oil is not a cold pressed. Physico-chemical properties of fenugreek seed oil and fatty acids composition of oil extract have shown respectively Table 1.10 [41], and Table 1.11 [36], on below. Chemical results show that fenugreek seed oil can be used as edible oil. Approximate PUFA content of oil is between 50-60 %. The amount and type of the fatty acids composition determine the quality of seed oil. Gas chromatography and mass spectrometry is the main

method for detecting of fatty acids. Besides, fenugreek seed oil used in perfumes because of its strong odour. It possesses volatile organic compounds, therefore it used in aromatherapy [28]. Plant-based oils are providing natural sources of n-3 fatty acids and strong antioxidants, for this reason, consumption of fenugreek seed oil can improve human nutrition.

Table 1.10. Physico-chemical properties of fenugreek seed oil.

Properties	**Values**
Ester value	190.25
Free fatty acid value (Oleic acid value) / 100 g oil	2.38
Saponification value mgKOH/ g of oil	195.0
Acid value mgKOH/ g of oil	4.75
Refractive index/ 37 C temperature	1.4640

At the present time, the demand for edible oils is increasing in order to be applied to functional foods. That is the reason fenugreek seed oil attracts the attention of scientists. According to the current investigation, oil extract from fenugreek seed has an antioxidant effect and it may be potential for use as food additives for the human diet. Usually, the organic solvent as hexane and petroleum ether is used to extract oil from seeds [41].

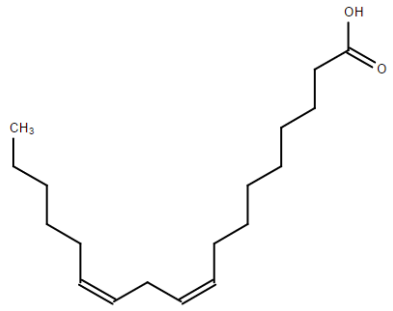
Conventional oil extraction technologies could be useful for laboratory scale, however, organic solvent extractions leaves behind toxic solvent residue. That is a time-consuming process and makes degradation of compounds because of high temperature. With the progress of science, several alternative methods could be used for extraction of oils from seeds. One of the innovative and effective methods is supercritical carbon dioxide extraction (SC-CO2). This method provides great opportunities for extraction natural oil. Comparing to the method of organic solvent extraction, SC-CO2 has a non-toxic effect on oil, non-flammable and easily removes from the extract [42].

Table 1.11. Fatty acid composition of fenugreek seed oil from extraction.

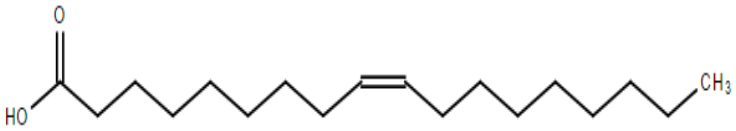
Name of fatty acid	**Approximate content (%)**
Linoelic acids	43.2
Alfa-Linolenic acids	22.0
Oleic acids	16.7
Arachiric acids	1.5
Steraic acids	4.5
Palmitic Acids	11.0

Scientists recently have determined that fenugreek oil has an antimicrobial and antibacterial activity against a microorganism. Effective inhibitor effect has found against on *Escherichia coli (bacteria), Staphylococcus aureus (bacteria), Aspergillus Niger (fungi) and Salmonella typhimurium* [41]. Because of the antimicrobial effect of fenugreek seed oil, it can be used in the food industry to improve the shelf life of food. There are not many studies have been completed about fenugreek seed oil until nowadays. For the further studies, we could say that edible oils from natural plants possess unique properties and it may be used together with new technologies on the biotechnological food industry.

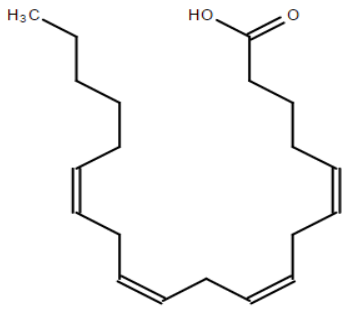
Common fatty acid derivatives present in fenugreek



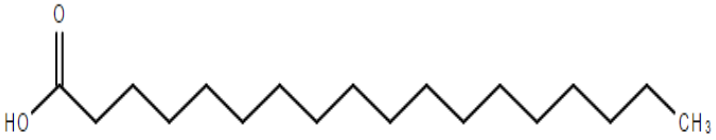
Linoleic acid



Oleic acid



Arachidonic acid



Steraic acid

1.4. Fermentation and hydrolysis of herbal plants

Fermentation is a process based on different species of microorganism and it has been applied to preserve foods since ancient time. With the progression of the technology showed up new preservation technique, therefore fermentation process diverted to be used different applications on foods. Essentially fermentation breaks eliminate undesirable substrates into appropriate compounds by the action of enzymes, thus process improves the nutrient values of foods [38]. Today the most

used fermentative products of the food industry are; cheeses, fermented milk, meat, vegetable, bread and alcoholic beverages. Recent studies proved that fermentation process improves biological features of plants and herbs. In more detail, fermentation effects on complex substances of plant compounds and changes them into compatible substances, this process associated with several biochemical changes, it related ratio of different nutritive value, bioactivity, and digestibility [39].

With the further development of the fermentation technology, through new and comprehensive researches on plant materials makes possibility to apply various plant active substances in the functional foods. On the Table 1.12 [64], shown that various changes of food properties caused by fermentation process [40].

Table 1.12. Changes in the result of food fermentation.

Description	**Change**
Hydrolysis of polymeric compounds as polysaccharides and proteins improve microorganism digestibility	Nutritional Value
Acidity increases, sweetness reduces by fermentation of sugars to the acids, bitterness may reduce by enzymatic activity.	Flavour
The production of volatile compounds such as fatty acids, aldehydes, esters, amines and ketones gives aroma	Aroma
Degradation (chlorophyll) and enzymatic browning can produce brown pigment, proteolytic activity	Colour
Changes of proteins and carbohydrates cause softened on products	Texture
Essential amino acids, essential fatty acids and protein	Enrichment with

Fermentation is one of the most useful techniques that produce active and non-toxic bioactive products from plant, it also stimulates the structural breakdown of plant cell walls, thus provides separation or synthesis different anti-oxidant compounds. Evaluation of bioavailability is one of the important parts of consumption plant, herbs extract and fermentation may compose of active components into low molecular weight substances. It has been investigated that fermentation may cause anti-oxidant activity of herbal plants by increasing total phenolic compounds of herbs. Especially, bacterial-mediated fermentation can accelerate the absorption ratio of flavonoids by constructive changes and that might increase bioactivity of active ingredients.

Recent studies focused on several experiments on fermented ginseng seed and the results showed that fermented ginseng seed was higher antioxidant activity and total phenolic content, comparing with unfermented seeds [43]. However, there is not much studies and information about

fermented fenugreek seeds, it could be a great source on innovative and promising for the future studies.

Table 1.13. Fermentation conditions for selected herbal plants.

Microorganism	**Herbal formulation**	**Fermentation medium**	**Solvent**	**Temperature and time**
B. longum, L. acidophilus, Leuconostoc mesenteroides	Codonopsis lanceolata	-	70% ethanol	30 C, 48 h
B. licheniformis	Rhizoma Atractylodis Macrocephalae	LB broth	Water	31 C, 24 h
L. fermentum, L. casei	Ssanghwa-tang	MRS broth, agar	Water	37 C, 48 h
Lactobacillus	Oyaksungisan	MRS broth	Water	37 C, 48 h
L. casei	Hwangryun-haedok-tang	MRS broth	Water	37 C, 48 h
Ganoderma lucidum	Ginseng	Ginseng medium	Water	RT for 37 days
L. acidophilus	Jaeumganghwa Tang	MRS medium	Water	37 C, 24 h
L. plantarum	Rhizoma Atractylodis Macrocephalae	Lactobacilli MRS broth	Water	37 C, 24 h
L. gasseri, Pediococcus pentosaceus, B. subtilis	Ginseng seed	MRS broth	Water	30 C, 24 h
Aspergillus niger	Ginkgo biloba leaves	10 g of solid medium and 16 mL of nutritive salt	Water	30-40 C, 6 days
L. brevis	Artemisia princeps Pamp	MRS agar plate	Water	30 C, 3 days

On basic, there is two type of fermentation has been applied on herbal plants. According to the substrate(s) used; Solid-state fermentation (SSF) and liquid fermentation (LF) types have been categorized. Both types of fermentation method have different properties and advantages. In solid-state fermentation, various microorganism grows on solid materials, and substrates are reduced slowly and used for a long time with controlled conditions by the absence of free liquid. SSF provides a couple of advantage such as higher fermentation productivity, well concentration of last product and effective on product stability. In LF microorganism demand a high amount of moisture and microbial growth occurs with liquid substrates. Especially liquid fermentation process is suitable for large- scale production [44]. On the other hand productivity and yields are important for the fermentation process. The temperature of fermentation, pH of the medium, properties of the solvent, incubation times and several factors are an effect on the fermentation process. In China, several studies have been reported on fermented herbal plants and seeds. In these report, several herbal plants and their optimal growth condition have shown in Table 1.13. [43].

1.4.1. Starch hydrolysis of plant material

Starch is a carbohydrate consisting of several glucose units bonded together with glycosides bonds. Starch is a polysaccharide and, generally produced by plants as an energy resource. It is important carbohydrate in human diets and starch contains foods such as potatoes, corn, rice, and wheat [118]. Fenugreek seed contains approximately 6 % percent of starch [14]. Pure starch is insoluble in cold water and alcohol, it has no taste, odourless, and consist of the linear and helical amylose. Starch presents in plant source occur granules form which could be a different size and physical characteristic from genius to genius. Starch molecules are not simple for consuming human digestive system, due to that reason amylases enzyme could break down the polymer into smaller sugar constituent which generally individual simple glucose units [119].

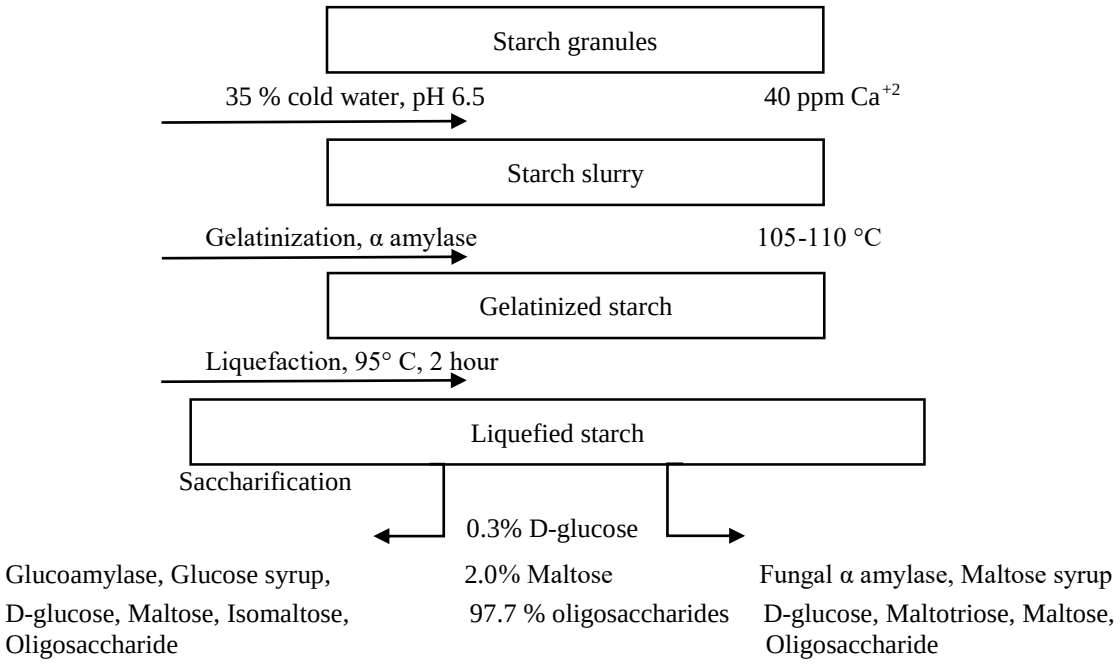


Fig. 1.4. Use of enzymes in processing of starch

Starch molecules resistant to influence by water because of the formation of hydrogen bonds together within different molecules, while the aqueous suspension of starch is heated, and then hydrogen bonds became weak by absorbing water starch molecules became swell. This process called generally gelatinization. Starch could be hydrolysed into simple carbohydrates by a different type of enzyme and acids. After hydrolysing process fragments are known as dextrin [118]. Acid hydrolysis has been used in past, nowadays enzymatic process replaced the hydrolysis. In the industry usually, starch hydrolysis process step and conditions are shown in Figure 1.4 [119]. After the process of

enzyme treatment starch granules converts simple sugar molecules; D-glucose, maltose, isomaltose and other oligosaccharides. That compounds could be used the good source for microorganism in the fermentation process.

1.4.2. Anti-oxidative effect of fermentation process

Microbial activity and oxidation are major factors that affect negatively on food spoilage. Those main factors cause rancidity and deterioration on foods. As a result, nutritional quality, colour, texture, and flavour of foods are changes. For the safety of the food, microbial activity and oxidation must be under controlled. At present time, food industry commonly uses chemical preservatives to prevent microbial growth. Scientifically proven that chemical additives or preservatives may cause possible health problems with consumption [45]. Due to these factors, consumers demand natural preservatives and substances for better quality foods.

Natural preservatives have a less toxic effect and more efficient on preserving food products from deterioration compared to the chemical additives. The plants, herbs, seeds and spices are important source of biologically active compounds and they are effective to stimulate cellular defences [46]. Antioxidants can quench reactive radical mediator formed during oxidative reactions. Various studies have shown that plant origin extracted bioactive compounds as, polyphenolic, phenolic compounds, flavonoids, alkaloids, vitamins, tannins and essential oils are possessing powerful antioxidant and antimicrobial properties. Most of the bioactive compounds extend shelf life and keep nutritional quality when added to the foods. That bioactive compounds are capable of adsorbing and neutralizing free radicals or decompose peroxides [47]. Most of the polyphenolic compounds extracted from leaves, fruits, seeds and roots. Those are a great alternative source of antioxidant and antimicrobial agents.

Previously, mentioned that about fermentation process that increases the phenolic content of plant materials [39]. In addition, several studies have been reported that fermentation enhances the antioxidant effect of several plants and plants products by increasing their $DPPH^{\cdot}$ radical scavenging activity on free radicals. On the plant cell walls, the structural breakdown is leading to deliver different antioxidant substances which capture free radicals through with fermentation process. Recent studies have been completed on soybean product [49]. That shows that several changes occur on macromolecular properties and antioxidant effect of products increased with the presence of fermentation-mediated substrates. Studies proved that relationship between polyphenolic compounds of plants extracted materials and antioxidant effects are closely related [48].

Fermented plant materials have more anti-oxidant activity and traditionally fermented herbal extracts used for the treatment of number of diseases. Other compound is flavonoids which increase during the fermentation. Flavonoids are quite effective to scavenge hydroxyl, peroxyl radicals and compress lipid oxidation. During the fermentation process, microorganism exposed to oxidative stress, therefore plant cells may improve protective mechanism containing enzymatic anti-oxidant, thus antioxidant effect contributed by the fermentation process.

The chemical composition of fenugreek seed suitable for fermentation. Seed is rich in bioactive, polyphenolic compounds [49]. Germinated fenugreek seed which possesses bioactive antioxidant components used broadly in food preparation. A lot of studies completed on anti- oxidant properties of fenugreek seeds extracts [4]. On the other hand, fenugreek seeds are fibrous, it has high content carbohydrates and fibre, in this way it could be an appropriate source for the microorganism. As a result of the studies proved that the fenugreek seed has a great source of antioxidants and antioxidant properties may increase by the treatment of fermentation process. For the future works, fermented fenugreek seeds provide promising opportunities for the functional foods application.

1.5. Functional food aspect of fenugreek

Fenugreek has been consumed in different countries for various purpose. It can be used as a supplement for foods, on various biotechnological application and hay, silage to livestock feed. From early times plants was known to cure diseases. In modern food technology, fenugreek may be used for many purposes. The crop similar like fenugreek is attracting the interest of food producers. Fenugreek or different modified fractions of fenugreek seed could be added to foods as baked good for better quality, on meat products to preserve, nutritional supplement or various functional food applications [50]. Fenugreek gum which present high amount of fenugreek seeds can modify various foods texture. Flavour components could be used to modulate organoleptic properties of foods. Soluble fibre can be used on yogurts, dairy products, and beverages to increase nutritional value. Modified powders of fenugreek seeds can be mixed with different kind of fruit juices and it can be formulated as capsules for used directly as a supplement [51]. The powdered seed might be more applicable on soups, cake mix and corn chips. Protein, fibre, total iron, and calcium value of white flour increases when mixed with 10 % of fenugreek flour [52]. This demonstrates that fenugreek can be associated to prepare with bakery goods for improving their protein amount or positive effect on their nutritional values. Previously, the impact of fenugreek flour on beef burger investigated. Fenugreek seed flour has been compared with soybean flour [53]. The burger that used fenugreek flour gave better results such a physiochemical quality criteria, microbiological quality, and content

of essential amino acids. We could say more example from previously studies, and for the further research, fenugreek is a source of inspiration on new food applications. On Table 1.14 shown studies related with functional food developed with fenugreek additives [50].

Table 1.14. Several functional food application reported with fenugreek

Functional food view of fenugreek	**References**
It has been reported that fenugreek seed rich in calcium, iron and p-carotene, fenugreek possess protein, lipid and cellulose starch.	[65]
Roundly half of the dry weight of fenugreek seed has edible and soluble dietary fibres (SDF).	[66]
It has been investigated that level of crude fibres increases while boiling of the fenugreek seeds, however loss of protein, sugars and ash content of seed.	[67]
It has been reported that protein and fat content of wheat which supplemented with the fenugreek mixture increases	[68]
When bitterness of fenugreek seed decreases while the processing, nutritional quality of germinated, soaked or raw fenugreek seed could be improved	[73]
It has been observed that supplemented Indian bread processed with higher temperature and longer fermentation time, phytic acid levels reduces, simultaneous *in vitro* increase calcium and iron amount	[69]
Physical and sensory properties and qualities of breads and cookies improved used with supplemented fenugreek flour on rice products	[70]
Fenugreek seed mucilage could be increase viscosity level of used material	[71]
It has been studied on eggs with fenugreek supplemented and oxidative stability of products improved	[72]
Fenugreek mucilage has been used in ice cream as a stabilizer	[73]

1.6. Methodological Aspect of Studies

1.6.1. Different systems for fractionation and extraction technology

Plants are a rich source of important bioactive compounds, because of that is reason extraction of those biologically active compounds are very important for the scientists [81]. Various technique and method have been used; simple traditional technologies as hydrodistillation, Soxhlet extraction, solid-liquid extraction and advanced extraction techniques as microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), ultrasound-assisted extraction (USE), enzymatic-assisted extraction (EAE). So far, usually conventional method has been

applied to the extraction of herbal plants and seeds. With the development of new extraction techniques especially environmentally green technologies which could contribute high yield activity of extracts or low toxic effects from solvents [80].

Evaporating alcohol from herbal plant extracts

For the scientists who analyse herbal plants, there are several problems with removing alcohol or solvents from extracts. Time-consuming and complicated steps are the main reason of removing process. The conventional method used for removing alcohol from extracts are hot water bath or steam water bath requires much time. Therefore new evaporation methods could be effective, fastest, most efficient and environmentally friendly way using a rotary evaporation method. Rotary evaporation is the equipment that removes volatile solvent from non-volatile substances. Basic figure of the rotary evaporator is shown in figure 1.5. Rotary evaporator reduces the pressure to lower the solvent boiling point, rotating sample to increase surface area and it heats the solutions. It gives great advantages to removes solvents from plant extracts [82].

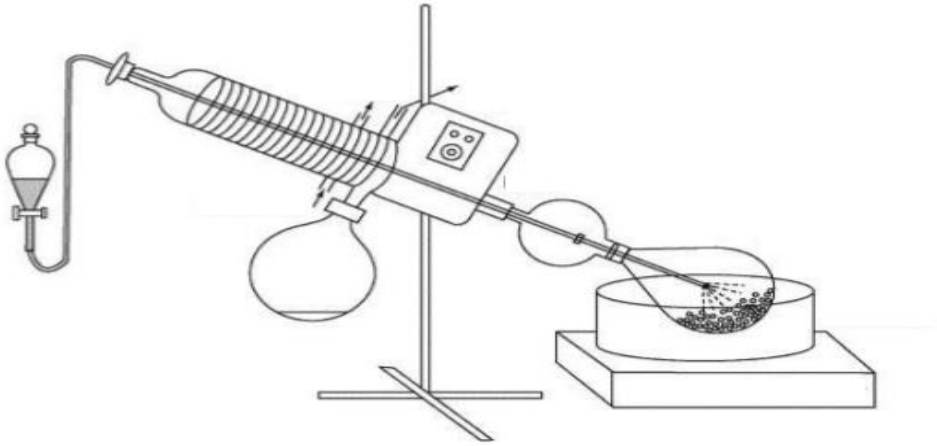


Fig 1.5. Basic figure of rotary evaporator

Soxhlet Extraction

Soxhlet extraction was designed specially to isolate lipids. Nowadays, it is used to separate biologically active compounds from different materials. Basically, the principle of that method; the solvent used depends on its polarity [83]. As the solvent boils the vapour passes through the condenser and the pure form of the solvent drops on the sample. Some disadvantages of this method are poor extracts of polar lipids, required a long time, and large volumes of solvents, therefore dangers of boiling solvents. Usually, hexane used to remove fat from materials. Ethyl acetate used for removing the phenolic compounds [84]. Removing fat from fenugreek seed and used by Soxhlet extraction

method is not efficient because of taking time and toxicity of solvents. Therefore, more environmental and effective methods can be used.

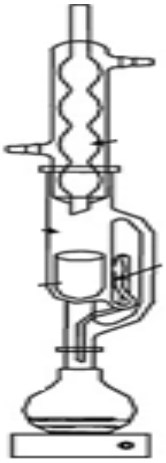


Fig. 1.6. Basic figure of Soxhlet apparatus

Supercritical Fluid Extraction (SFE)

The main system of SFE consists of a CO_2 pump, CO_2 tank, co-solvent vessel and pump, an oven that contains extraction vessel, heater and a controller to maintain the high pressure inside the system by a trapping vessel [85]. Carbon dioxide is ideal solvent for supercritical fluid extraction because of its nontoxic, non-flammable, noncorrosive and affordable properties. The critical temperatures and critical pressure depend on extract material. Various yield rates and the result has been observed by different plant materials which are extracted. SFE has been used commercial natural food products; spices, flavours, coffee and some vegetable oils [86].

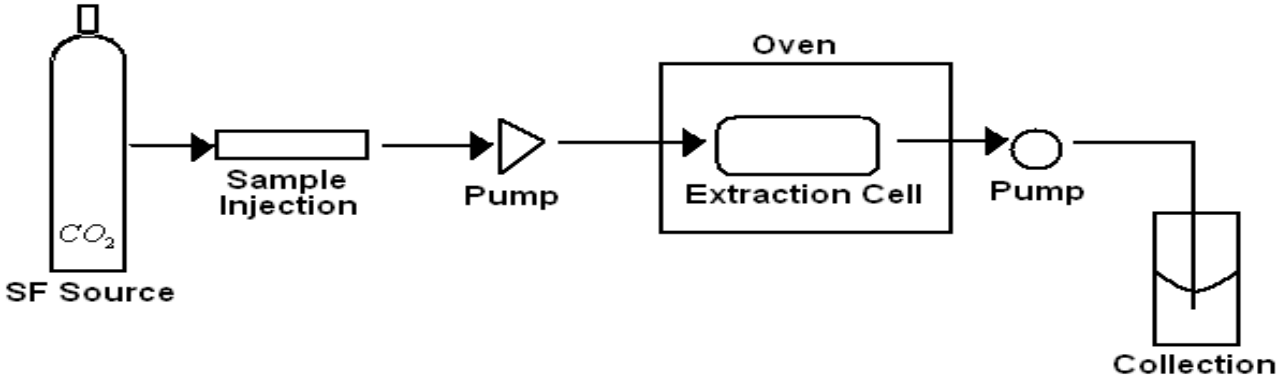


Fig. 1.7. Basic principle of Supercritical fluid extraction scheme

Because of the advantage of SFE, oil from plant materials as fenugreek seed could be extracted with high yield rate and purity of oil [87]. Comparing the conventional methods as soxhlet extractions,

SFE is providing a great opportunity, not only identify and analyse compounds, besides commercially could be used for further studies.

Plate count technique to determine microorganism

One of the main techniques is plant counting to determine the number of living cells in the sample. There are several step and technique and all should be completed carefully because samples could be easily contaminated from the environment. An aseptic technique which includes holding tubes at an angle, using sterile pipettes and working with flame must be used for effective and accurate results [90]. Diluting of the sample is necessary because of the reason that per millilitre of the sample might contains millions even billions of microorganism. After dilution, agar plate prepared by appropriate media. Media depends on microorganism which we want to growth it. Once the plates have been prepared then moved to incubation process. Incubation times and temperatures are depending on various microorganism [91]. The last step is determination and counting microorganism carefully. Countable microorganism colonies are between 30 and 300.

Lyophilization / Freeze- drying process

Freeze drying is a process which could remove water from the sample by the presence of very low temperature. Mainly process consist respectively two steps; sublimation and desorption. Firstly the material was frozen and then it exposure high vacuum after frozen liquid sublimes. The principle is based on physical status. When the atmospheric water pressure is lower than partial pressure, the ice straight converted into water vapour [88]. There are several advantages of freeze drying process. Oxidizable components are well protected under vacuum circumstances and because of the high amount of water removal from materials could preserve foods long time. Under low temperature microorganism could not grow and enzyme activity could be less. Therefore product can be storage long time after freeze drying process. On the other hand, Lyophilization is quite expensive process and while processing volatile compounds could be removed by high vacuum [89]. Even so with the development of new application on foods, the freeze drying process is promising for further research.

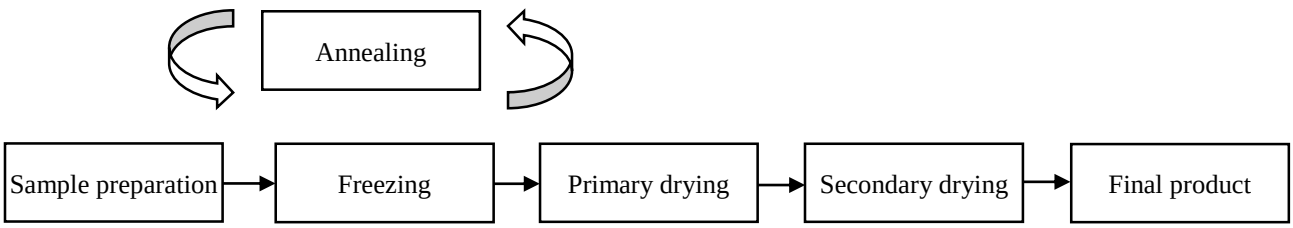


Fig.1.8. Lyophilization cycle

1.6.2. Determination and quantification of various compounds

The determination and quantification step is very important to get know properties of fractions. There are a huge number of factors influencing their recovery, especially when food samples are analysed. Various determination methods were used in the analysis of foods and various methods are; solid-phase microextraction, solid-phase extraction (SPE) [100], ultrasonic extraction, Soxhlet extraction, liquid-liquid extraction and blue cotton. Solid-phase extraction method commonly used for double or singe extract and clean-up procedures [101].

Gas chromatography-mass spectrometry (GC-MS) [147], liquid chromatography with mass spectrometric (LC-MS) [150] methods are suitable to separate, identified and quantified individual components from modified fractions. Various chromatographic methods has been applied to determine on herbal extracts from plants. Thin layer chromatography is rapid, easy method to isolate natural and synthetic compounds. TLC has ability to detect a wide range of compounds by using reactive spray reagents [74]. GC is useful to analyse volatile compounds. In mobile phases gas could transmitted sample into a vapour state through stationary phase. Low viscosity of gas allows for the use of long columns and fast analysis by high gas flow rate is advantage of using GC. Disadvantages of GC is not applicable on non-volatile compounds and requires capable operators [75]. Another method is High Pressure Liquid Chromatography (HPLC).

HPLC is a method which developed of column chromatography and application areas is more extended incudes pharmaceutical industry [76]. Main detectors used in HPLC are; Refractive index (RI), Ultraviolet/Visible (UV/Vis), evaporative light-scattering (ELS), MS and Fluorescence detector. HPLC columns are very small sized (3, 5, 10 μm) and tightly distributed. Solvent selection depends on the mixture of components and interested compounds. Methods can detect compounds depends on samples purity and variety [77]. Each methods for the identification of fractions have their own advantages and disadvantages. LC-MS method are very sensitive and particular. It could determine large number of individual chemical compounds present in the extract. MS has low cost of operation and high efficiency on separation [102-104].

One of the advanced technique is headspace gas chromatography technique. It is used for to analyse volatile organic compounds. The popularity of headspace technique day by day increases and commonly applications include; beverages and food products, polymers and plastic analyses, flavour compounds and perfumery [78].

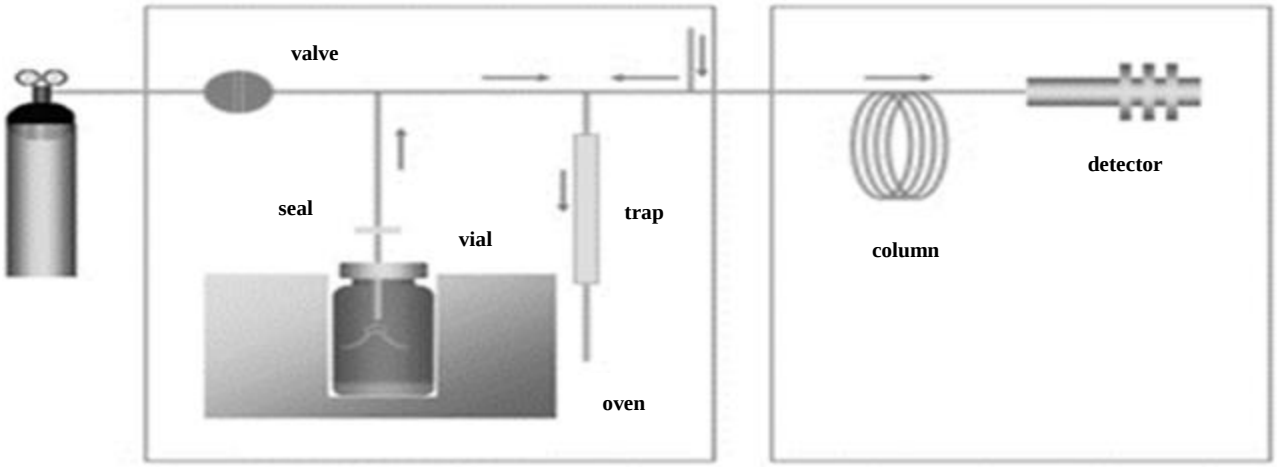


Fig 1.9. Basic scheme of head space technique

Headspace equipment consists of a hollow dome which forms the hermetic seal. The basic principle is inactive gases are passed through the object which contains space and vacuum are based on removing odour compounds from headspace. After the compounds captured between cold surfaces, adsorbent materials, and solvent traps, lastly, the sample could be analysed gas chromatography, mass spectrometry, or Carbon-13 NMR technique. Samples contain high-molecular-weight and non-volatile compounds that could remain in the GC, thus it causes poor analytical results [79]. Headspace technique prevents from undesirable volatile compounds, time-consuming and high cost. Other advantages of this method are easily operated and capable of analysing variation of sample matrices.

2. MATERIALS AND METHODS

2.1. Reagents and materials

All reference compounds used as standards were obtained from Toronto Research Chemicals (Toronto, Canada). The list of used materials and reagents shown in Table 2.1. Fenugreek seed was supplied local market in Antalya, Turkey, and it was kept in tightly closed, dry sterile plastic bag, in dark, well-ventilated room. Fenugreek seed fraction treated by alfa-amylase and glucoamylase enzymes were purchased from UAB "Baltijos enzymai" in Vilnius, kept in the fridge (4°C). Lactobacillus casei bacteria was prepared in Department of Food Technology according to the previously improved procedure [92].

Table 2.1. Material and reagent used for analyses

Reagent/ Material	**Characteristic**	**Supplier**
Acetic acid	-	Sigma-Aldrich (Steinheim, Germany)
Methanol	-	Sigma-Aldrich (Steinheim, Germany)
Acetonitrile	-	Sigma-Aldrich (Steinheim, Germany)
Diethylene glycol	-	Sigma-Aldrich (Steinheim, Germany)
Ethly acetate	-	Chempur (Poland)
Sodium hydroxide	-	Merck (Darmstadt, Germany)
Hydrochrolic acid	-	Merck (Darmstadt, Germany)
Ammonium hydroxide	25 %	Merck (Darmstadt, Germany)
Trifluoroacetic acid (TFA)	-	Merck (Darmstadt, Germany)
C18 columns	-	Phenomenex (Torrance, CA, USA)
HCl	35-38%	Chempur (Piekary Slaskie, Poland)
Dichlormetane, pentane, hexane	-	Sigma-Aldrich (Steinheim, Germany)
Acetone, methanol, hexane	Analytical grade	Sigma-Aldrich (Poole, UK)
Ethanol	96.3 %	Stumbras (Kaunas, Lithuania)
Carbon dioxide gases, nitrogen gases	99.9%	Gaschema, Jonava region, Lithuania
Catalytic tablet	K_2SO_4, $CuSO_4$	Sigma-Aldrich (Steinheim, Germany)
2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)	($ABTS^{\bullet+}$)	Fluka Chemie (Buchs, Switzerland)
2,2-Diphenyl-1-picrylhydrazyl hydrate	$DPPH^{\bullet}$, free radical, 95%	Fluka Chemie (Buchs, Switzerland)
3,4,5-trihydroxybenzoic	Gallic acid, 99%	Sigma-Aldrich (Steinheim, Germany)
2-(3-hydroxy-6-oxo-xanthen-9-yl)	Fluorescein (FL)	Bornem (Belgium)
MRS broth media	-	-
Alfa-amylase, gluco-amylase	-	Vilzim AMY (Vilnius, Lithuania)
StableFlex silica fiber 60 μm	Polydimethysiloxane/ divinlybenzene coating	Bellefonte (PA, USA)
6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid	Trolox, 97%	Sigma-Aldrich (Steinheim, Germany)

2.2. Determination of fenugreek seed chemical composition

Protein, fat, moisture, ash and carbohydrate amount of samples (fenugreek seeds, and FPF) were determined by used appropriate method and described below.

2.2.1. Protein content

Protein content has been completed by using the Kjeldahl method. It was determined with automatic Kjeltec equipment. Three different samples were applied to obtain protein content of fenugreek seed. 1 gram of seed samples (ground) was weighed and added into the special Kjeldahl flask, then filled with 20 ml concentrated H_2SO_4 together with catalyst tablet (3.5 g K_2SO_4, 0.4 g $CuSO_4$). Samples were mineralized while solution in the flask became transparent. The heating time was approximately 95 minute and heating intensity 55 %. The solutions were distilled with the automatic steam distillation system. All solutions were distilled, automatically filled NaOH and H_3BO_4 with the steam intensity 85 %. Distillate solutions were collected in the flask, and then added Tashiro indicator, titration was completed with 0.01 N HCL until the colour turns into grey-violet colour. A control sample was done with concentrated H_2SO_4 instead of a sample. Nitrogen content was calculated by the following formulation:

$$x = \frac{0.0014 \times A}{m} \times 100; \%$$

Where: A – 0.1N HCL amount, used for distillate titration, ml; m – sample weight, g; 0.0014 – nitrogen amount equivalent 1 ml 0.1 N HCL. Protein material amount was calculated by multiplying the amount of nitrogen from the conversion factor 5.7.

Same method were used for the protein determination of FPF.

2.2.2. Lipid content

Three different seed samples were analysed to determine lipid content by used Soxhlet method. Samples (0. 1-0. 2 mm) of fenugreek seed was dried after moisture content determination and between 2.9-3.0 gram seed samples were placed to filter paper and rolled up tightly. The samples were carried out Soxhlet extraction used with hexane solvent and then waited 6 hours at 70 % temperature. Soxhlet extraction procedure was described previously in 1.5.2 paragraph. After the process of extraction samples were dried in thermostat (80 C) until all solvent evaporated and then weighted with 0.0002 g accuracy. The lipid content of samples was calculated by the following formulation:

$$x = \frac{(a - b) \times 100}{m}; \%$$

Where: a – sample weight with filter paper before extraction, g; b – sample weight with filter paper after extraction, g; m – sample weight taken for extraction, g.

2.2.3. Mineral content

Three samples of (0.1-0.2 mm) ground fenugreek seed and three samples of fenugreek fraction were analysed to determine mineral content. The crucibles were dried and weighted with 0.0002 g accuracy. Samples were weighed between 3- 4 gram, and then placed into crucibles. Samples were added on an electric hot plate, heated until the smoke has stopped to forming. After that, crucibles were transferred to the muffle which internal temperature has been set 600-650 °C. Process of combustion have lasted about 17 hours. Samples were burned until two consecutive weightings differ 0.0001 to 0.0005 g. Mineral content was determined by the following formulation:

$$x = \frac{(m_2 - m) \times 100}{m_1 - m}; \%$$

Where: m – crucible weight, g; m_1 – crucible weight with sample, g; m_2 – crucible weight with burned sample/ g.

2.2.4. Moisture and hydrocarbon determination

Before the measurement of moisture content of fenugreek seed and fraction of seed, the glasses with cap and rod were heated and weighted with 0.002 g precision. Three samples of seed and fraction were weighted (approximately 3 grams). After that samples were stirred and dried in the oven at 100-105°C. The samples were periodically weighed every 3 hours until the results of weightings varied between 0.001-0.005 g. Each times samples were placed into a desiccator for 20 minutes to cooled samples. Moisture content was calculated by the following formulation:

$$x = \frac{(m_1 - m_2) \times 100}{m_1 - m}; \%$$

Where: m – glass with cap and rod weight g; m_1 – glass weight with sample before drying g; m_2 – glass weight with sample after drying, g.

Hydrocarbon determination

The carbohydrates content was calculated by subtracting the previous components from a hundred. The experiments were made in triplicates, and then the means were calculated. Total carbohydrates amount (%) = 100 - moisture (%) - protein content (%) - Crude fat (%) - ash (%), total carbohydrates, protein, crude fat, and ash content were fresh weighted and determined as total carbohydrates in % [135].

2.3. Fractionation and extraction process of fenugreek seed

In this study, we obtained four main fractions: Fenugreek ethanol fraction (FEF), fenugreek oil fraction (FOF), fenugreek fermented fraction (FFF), and fenugreek powdered fraction (FPF). General process line is shown in Figure 2.1, and in 2.3.1 paragraph. At first, the fenugreek seeds chemical compounds analysis were completed with most common methods used according to the AOAC (1995) [158], and later fractionation process was applied. Dry fenugreek seeds were ground to 1.0-2.0 mm particle size with IKA M20 laboratory scale grounder, filtered to 1.0 mm (with sieve), prepared for the ethanol extraction, later ethanol extract process applied and method described in detail in 2.3.1, paragraph. The extracts and seed residues were separated. Ethanol extracted dry fenugreek seed was prepared for analysis by passing through the following procedures respectively; filtration, evaporation, and freezing. The seed residue which remained from ethanol extract was dried with the appropriate conditions, placed in thermostat (60°C, 4 hours), prepared to the next process, then Supercritical CO_2 extraction process was applied properly to obtain oil from the seed. SFE-CO_2 extraction method and process described in detail in 2.3.2, paragraph. After the SFE-CO_2, 1719 gram seed sample was placed in the multipurpose tank, mixed with water (V/15V solid solvent ratio) and prepared suitable condition for fermentation application. Fermentation application described in detail in 2.4 paragraph. When the fermentation application finished, two different fractions(FFF, FPF) was collected and applied process respectively; cooling, freezing, lyophilization, and stored until used. Freeze drying process described in detail in 2.3.3, paragraph. End of the process four fractions was analysed, all analyse method described in method part below, and the yield of the process were calculated and expressed as g/100 g DW.

2.3.1. Ethanol extraction of seed

Five different samples were used for ethanol extraction. Extraction was applied on at the room temperature (25°C) with 96. 3% concentrated ethanol by (1/1 v) solid/ solvent ratio. Fenugreek seeds were grounded to 1,0 mm size and placed into the 750 ml flask. Each time, 300 gram ground fenugreek seed (totally 1950 gram seed) were placed in a flask and extracted two times with the 300 mL of ethanol by constant shaking during 2 hours, after that the extracts were filtered with the laboratory filter paper. Filtration process was completed with a vacuum pump and each time it took around 1 hour. After the filtration seed extracts with ethanol was placed into the flask to the vacuum rotary evaporator and seed residue has been collected to be used for next process. Evaporation process was completed with the respective parameters; bath temperature has been set between 35-45°C, vacuum

pressure between 90-120 mbar. Dry extracts and ethanol were separated. The extracts were stored in a freezer until used and ethanol was gathered to be used for another time.

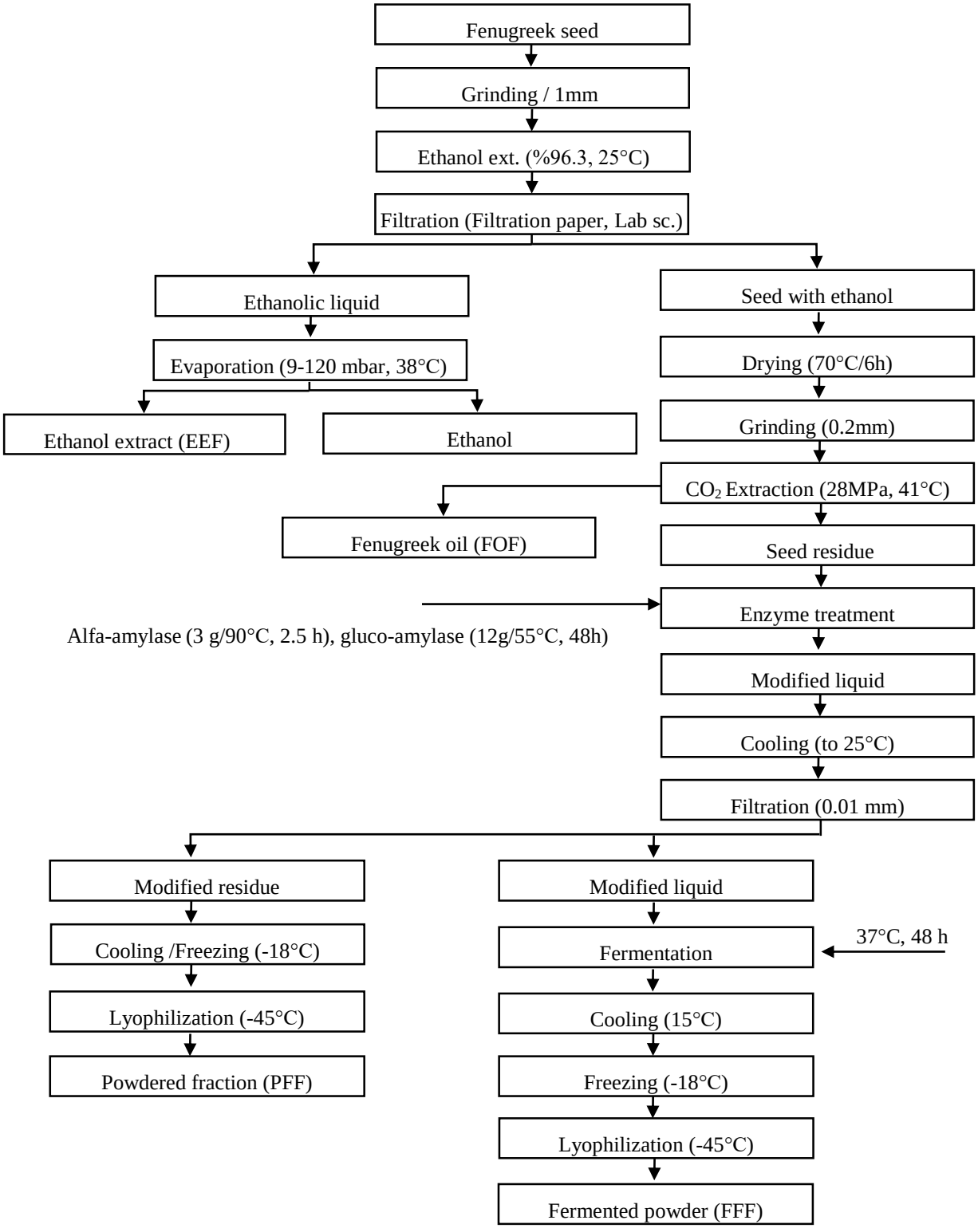


Fig. 2. 1. General flow chart of fenugreek seed fractions process

2.3.2. Oil extraction of seed

Supercritical CO_2 extraction (SFE-CO_2) was applied in a supercritical fluid extractor Helix extraction system (Applied Separation, Allentown, PA, USA). The basic principle of the SFE-CO_2 apparatus shows in Figure 1.7, in the literature review. General flow chart of oil extraction process shown in Figure 2.2. Firstly seed has been ground to 0.2 mm with ultra-centrifugal mill ZM 200 machine. The basic principle of the machine; the feed material passes through the hopper onto the rotor, centrifugal acceleration throws it outward with huge energy and wedge-shaped rotor teeth moving at a high speed, and then material finely ground between the rotor and the ring sieve. Speed has been set 6.000 min^{-1}, process time was 2.5 hours. 1850 gram dried, ethanol extracted fenugreek seeds samples (totally 5 different samples) which collected from ethanol extraction process have been grinded and after placed into a in a 50 cm^3 cylindrical extractor, 14 mm inner diameter and 320 mm length. Round balls were placed on the top cylinder to avoid particle release to the system. The volume of CO_2 was measured by a digital mass flow meter in standard litres per minute (SL/min) at a standard state (P_{CO2}= 100 kPa, T_{CO2}= 20°C, ρ_{CO2}=0.0018 g/mL). The process consisted of dynamic and static extraction steps. Extraction pressure, temperature and time were set manually and respectively; 28.5 MPa, 41°C and 200 min extraction time. After that, the extract (seed oil) was kept in the plastic sterile bag until used. Seed residue was kept in plastic sterile bag until to be used for next process. Extraction yields were expressed as g/100g DW.

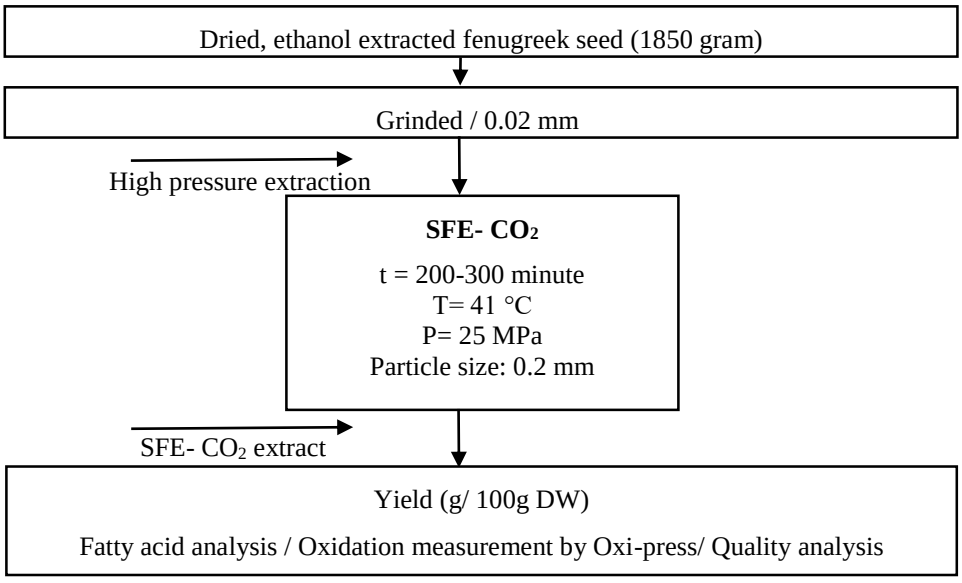


Fig 2.2. SFE-CO_2 process flow chart to obtain fenugreek oil

2.4. Fermentation application on modified fraction of fenugreek

2.4.1. Enzyme treatment of modified liquid fraction

Enzyme treatment was applied in a Multi-Purpose Fermentation vessel, 20 litre capacity (MPV220-FE model, OMVE, Netherlands B.V.). The vessel designed for pilot fermentation projects in a fast, easy and controlled way. The system included PLC touch screen, it gives that possibility to adjust parameters and could control all process step fully automatically. Approximately 1700 gram extruded seed was placed in the equipment together with the (V/15V solid solvent) litre of tap water by constant mixing while temperature increased. When the temperature of the vessel was reached 90 °C, 3 grams of alfa-amylase enzyme was added (0.1-0.2 kg/ton) according to amount of usage. The sample was mixed very slowly around 2.5 hours in the tank and every 30 minute sample was taken for pH analysis. Later sample was cooled to 55°C, and then 12 grams gluco-amylase enzyme added. Sample was mixed gently, 48 hours sample was incubated with 45-55 °C temperature and periodically 5-10 ml sample was taken for control the parameter. After the enzyme treatment samples were filtered due to the reason separate big particle from the liquid and prepared for the fermentation.

2.4.2. Microorganism application and determination of fenugreek fraction

Solid state fermentation (SSF) was carried out in a Multi-Purpose Fermentation vessel, 20 litre capacity (MPV220-FE model, OMVE, Netherlands B.V.). Lactobacillus casei were prepared at the Department of Food Technology of Kaunas University of Technology (KTU, Kaunas, Lithuania). Firstly mass ratio of 2% (of total water and seeds) of freshly prepared, the lactic acid bacteria culture was mixed with sterile water and then mixed with the seed fraction. Fermentation was done at the optimal temperature and conditions (37°C and 48 hours) for Lactobacillus growth. Properties of the fermented products such as; pH (6, 8, 24, 48h), total titratable acidity (TTA), microorganism growth and total amount of microorganism were determined during and after fermentation. Total titratable acidity (TTA) of the fermented products and bread crumbs was measured according to the method described in AACC [54]. The TTA value was expressed in millilitres of 0.1 M NaOH solution used per 10 g of sample to obtain pH=8.5. The specific volume of the bread samples was evaluated as described in AACC [54].

Microorganism determination was completed on sample before and after fermentation according to the plant count technique by estimating their number used with MRS broth medium. (Liofilchem, Roseto degli Abruzzi, Teramo, Italy). Plates were prepared and incubated for 5 days under anaerobic conditions, and then placed in thermostat. The number of bacteria was expressed as

a decimal logarithmic value of colony forming units per gram (log CFU/g). The experiment was done triplicate.

2.5. Freeze Drying of modified seed fractions

Lyophilization process has been applied on two different fractions (FFF, and FPF) obtained from fenugreek seed. Seed fractions which used for freeze drying were after the process respectively; SFE-CO_2 extraction, fermentation and enzyme applications on modified liquid of the seed. After the SFE-CO_2 extraction process, the seed was mixed with water, then enzyme treatment has been applied on modified fraction, and then the liquid was filtered. After filtration, modified residue of the fraction was left in the freezer (-18°C) for use on lyophilization process. Other modified liquid fraction was kept in the freezer (-18°C) for use in lyophilization process after the fermentation process applied on modified liquid. Freeze drying process was applied in Freeze Drying Plant Sublimator 15 (ZIRBUS Technology, Germany) system. Two different fractions and approximately 25 kg of modified seed materials were placed on the shelf (300×400 mm) with the configuration of 5 shelves / 80mm distance, 6/65, 7/55, 8/45. For each shelf were placed around 500 grams of samples due to the reason of high content water mixed with the fractions and high efficiency drying process.

The parameters for the process were; -45, -55°C condenser temperature, -18, -25°C product temperature at beginning and process time was between 12-20 hours. After lyophilization process, powdered form of samples was weighed and kept in the sterile plastic bag until used for analyses. Process yields were expressed as g/100g DW.

2.6. Analysis of fenugreek seed fractions

Various analyses has been completed on four fractions (FEF, FOF, FFF and FPF). The method used for analyses described below in the detail.

2.6.1. Analysis of fenugreek chemical fractions composition from headspace

Fenugreek seed fractions were analysed various methods. GC/MS analysis completed for FEF, FFF, FPF fractions. Volatile compounds were analysed by a headspace (HS) technology by using solid-phase dynamic extraction (SPDE) [95]. Two mL of samples were placed in 20 mL vials ,and headspace volatiles were sampled by HS-SPDE under the following conditions: pre-incubation time 2 minute; syringe temperature 76 °C; incubation temperature 70°C; extraction strokes 50°C; extraction fill/ eject speed 40 µL/s; desorption gas volume 500 µL; pre-desorption time 30 s; desorption flow speed 15 µL/s. The trapped compounds recovered from SPDE needle containing 90% dimethylsiloxane and 10% activated carbon directly into the GC injector by thermal desorption and

analysed by GC/MS (Agilent Technologies 6890N/5973, China). The volatile compounds separated by using a HP-5 capillary column (60 m length, 0.32mm id, 1µm film thickness). The temperature was set from 40°C to 240°C. All compounds were identified by their retention index and the comparison of their MS with library spectra present in Wiley and NIST databases.

2.6.2. Determination of the total phenolic compounds of fractions

The content of total phenolic compound on FEF and was determined by Folin-Ciocalteu method (1999) [96] with some modifications. It was prepared; calibration curve 1 mL aliquots of 0.024, 0.075, 0.105 and 0.3 mg/mL Gallic acid solutions were mixed with 5 mL of Folin- Ciocaleu reagent and 4 mL (75 g/L) sodium carbonate solution. The absorption was performed approximately one hour at 20 °C at 765 nm and the calibration curve was completed. 1 mL of FEF was (0.01% conc.) was mixed with the same, and after one hour the absorption was measured for the determination of phenolic content. The definitions were applied triplicate and the total content of phenolic compounds, C (mg/g) in similar extracts in Gallic acid equivalents (GAE) was estimated by the following formula:

$$C = c \times V \div m$$

Where: c- the concentration of Gallic acid established from the calibration curve, mg/mL; V- the volume of extract, mL; m – the weight of pure plant extract, g.

2.6.3. Radical Scavenging assay ($ABTS^{\bullet+}$, $DPPH^{\bullet}$)

The $ABTS^{\bullet+}$ scavenging assay

The $ABTS^{\bullet+}$ assay was carried out by the method of Re *et al*, (1999) [128] with some modifications. The phosphate buffered saline (PBS) solution (75 mmol/L; pH 7.4) was prepared by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of distilled water. The $ABTS^{\bullet+}$ solution was prepared by mixing 50 mL of $ABTS^{\bullet+}$ (2 mmol/L PBS) with 200 µL $K_2S_2O_8$ (70 mmol/L) and before the used, it allowed the mixture to stand in the dark at room temperature for 15-16 h. The active solution was prepared by diluting the $ABTS^{\bullet+}$ solution with PBS to get the absorbance of AU 0.700±0.010 at 734 nm. To a 1500 µL of working $ABTS^{\bullet+}$ radical solution 25 µL of sample (200-4000µg/mg) or MeOH (blank) were added, mixtures left in dark for 2 hours and absorbance was restrained at 734 nm.

$TEAC_{ABTS}$ of extracts were calculated by means of dose-response curves for Trolox. Extract:

$$y = 0.0659x + 0.2274, R^2 = 0.9989$$

The DPPH• radical scavenging assay

The DPPH• assay was carried out by the method of Brand & Williams (1995) [96], with some modifications as follows. To a 1000 µL of a ~89.7 µmol/L (final absorption adjusted to 0.800±0.010 AU at 517 nm) DPPH• methanolic solution 500 µL of sample (10-1000µg/mg) or MeOH (blank) were added. All mixtures were left in dark absorbance was measured after 2 hours at 517 nm.

$TEAC_{DPPH}$ of extracts and solid samples (before and after extraction) were calculated by means of dose-response curves for Trolox. Extract:

$$y = 1.3284x + 1.8618, R^2 = 0.9974$$

2.6.4. Determination of fatty acid methyl ester (FAME) of fenugreek seed oil

Previously fenugreek seed oil was extracted with SFE-CO_2 and determination of fatty acid composition were completed. A 0.5 gram of fenugreek seed oil was taken in 50 mL test tube and 4 mL of sodium hydroxide (prepared by mixing sodium hydroxide in methanol (0.5N) was added and the solution stirred vigorously using vortex stirrer for 20 seconds. Esterification was performed in a boiling water bath and heated the flask to boiling and reflux for 5-15 minutes to allow all the oil to dissolve, ant then used a pipette added 5 mL of hexane to the flask by refluxing for 1 minute. The sample was removed and cooled. After it carefully used a pipette and removed and aliquot of the hexane phase and transferred it to a 2 mL GC vial. A later sample was diluted approximately 1:10 with hexane (100 µL of sample: 900 µL of hexane), used for analysis. Aliquots of 1 µL FAME was injected and peaks were saved for their respective retention time and areas by the data processor unit of the GC.

FAMEs were analysed on an HRGC 5300 (Mega Series, Carlo Erba, Milan, Italy) equipped with a flame ionization detector and 100 m length 0.25 mm (id), 0.20 mm film thickness fused silica capillary column SP^{TM} – 2560 (Supelco, Bellefonte, PA, USA). Analysis parameters were as follows: injection temperature 220°C; detector's temperature 240°C; split ratio 100:1; oven temperature was programmed in three ramps from 80°C to 135°C at 4°C / min, and from 185°C to 240°C at 4°C/ min and held isothermal for 5 minute; carrier gas, helium at a flow rate of 20 cm^3/s. The compounds were identified by comparing their retention times with those of commercial FAME mixture. All solvents and standards were of analytical grade. (Sigma).

2.6.5. Measurement of oxidation properties

Measurement of the oxidation induction period of fenugreek seed oil was applied with the Oxi-press apparatus (Mikrolab, Aarhus, Denmark). The samples were prepared after the SFC-CO_2 extract of fenugreek seed. Oil placed out from the freezer (-18) to the room temperature and waited to be resolved. Five grams of seed oil were placed in a reactor tube and thermostat was set at 110°C under with oxygen atmosphere at 5 bars on Oxi-press apparatus. Pressure changes occurring due to the absorption of oxygen consumed for oil oxidation were recorded. Pure rapeseed oil was used as a control. Results showed induction period (IP, h).

2.6.6. Physicochemical characteristics of FOF

Peroxide Value of Fenugreek seed oil (FOF)

Peroxide value has been done on FOF. 1 gram of oil were placed in 250 ml flask, 10 ml of chloroform and 10 ml of Glacial acetic acid was added and mixed. 1 gram of KI-solution was added and placed 10 min in dark place. After 100 ml distilled water was added into a sample, 1% of starch solution added before the titration. At the end, sample was titrated with the 0.1 N sodium thiosulfate. An experiment has done triplicate. Peroxide value expressed according to the formula:

$$p.v. = \frac{(V - V1) \times 0.01269 \times 100}{m} (\frac{g}{100g} fat)$$

Where: V; 0.1 N $Na_2S_2O_3$ used for titration of test sample, V1; used for titration of the blank test, m; fat amount used, 0, 1269; iodine content, consistent 1 ml 0.1 N $Na_2S_2O_3$

Iodine Value of Fenugreek seed oil (FOF)

Iodine value has been completed on FOF. 0, 3-0, 4 gram fat was placed in a flask. 30-40 ml of 96% ethyl alcohol was added. After that 25 mL 0.2 N spirit of the iodine solution and 50 mL of distilled water was added. The sample was placed 5 min in dark place. Before the titration 0.5mL 1% starch solution added and sample was titrated with 0.1 N sodium thiosulfate. The experiment was done triplicate and iodine value was expressed according to the formula:

$$X = 0.01269(a - b) \times 100/m$$

Where: 0.01269; iodine number, compatible 1mL 0.1N $Na_2S_2O_3$ solution g/mL; a- 0.1 N $Na_2S_2O_3$ used for titration of blank sample, b- 0.1 N $Na_2S_2O_3$ used for titration of test sample, m- amount of fat, g.

2.7. Statistical analysis

All values and amount were expressed as a mean ± standard deviation. Standard deviations were calculated using spreadsheet software (Excel®) and, the correlation coefficients (R) to determine the relationship between radical scavenging activity, total phenolic content and formation of samples were calculated using MS Excel® software (CORREL statistical function).

3. RESULTS AND DISCUSSION

3.1. Chemical composition of fenugreek seeds

The protein, fat, mineral, moisture and carbohydrate content for the fenugreek seed presented in Table 3.1. The protein content of fenugreek seed was 29.11%. This amount was higher than reported by Sulieman (2008) [41] who determined 28, 55%, and Jani *et al.* (2009) who found 25.4% value of protein obtained by fenugreek seeds. Işıklı & Karababa (2005) [106] have reported that protein content of fenugreek seed various between 20-30 %. Pasricha & Gupta (2014) [61] reported 24.06% protein content of fenugreek seed. According to the Meghwal & Goswami (2012) [106] fractions of fenugreek seed protein (lysine) seed is a higher amount than soybean proteins. Srinivasan (2006) [16] investigated that, cooking process was not affected by changes the quality of fenugreek seed proteins. Nasri & Tinay (2007) [107] reported, a protein of fenugreek seeds are more soluble in alkaline and acidic conditions comparing to the condition neutral pH, the protein concentration of fenugreek seed has high oil and water absorption capacity and the bulk density is 0.66 g/ ml. Protein content of fenugreek seeds depends on different growth conditions; the climate of place, environmental differences, mineral, and composition of soil [105]. Proteins are very important for plant metabolism such a metabolic properties, structural, and physical characteristics. Besides, the plant origin proteins and amino acids have main compounds for human metabolism.

Table 3.1. Chemical composition of fenugreek seed

Index	**Amount, %**
Protein content	29.11 ± 0.89
Fat content	6.57 ± 0.49
Mineral/ ash content	4.05 ± 0.03
Moisture content	5.25 ± 0.72
Carbohydrate	55.02 ±0.12

The fat content of fenugreek seed was 6.57%. This amount was lower than reported by Sulieman (1995) [105], and Srinivasan (2006) [16] who reported respectively; 8.04% and 7.5%. Lee EEL (2006) [15] investigated that fenugreek seed lipids contain 84.1 % neutral lipids (the main composition is triacylglycerol), 10.5% phospholipids, and 5.4% glycolipids. Skaltsa (2002) [14] reported that fenugreek seed contains high an amount of diosgenin as a lipid steroid. Diosgenin is a very important compound for the pharmaceutical industry and it is used the raw material on natural medicine which stimulates sex hormones [14]. The fat content of natural plant material depends on various growth

conditions. Usually, the fat content of the plants may greatly differ from the anatomical part of the plant. Ambasta (2000) [109] was determined several physico-chemical characteristic of fenugreek oil: acid value 1-2, saponification value 178–183, unsaponifiable matter 3.9%, and iodine value 115.

The ash/ mineral content of fenugreek seeds was found to be 4.05%. This value was higher than reported by Sulieman (2008) [41] who reported 3.20% and lower than recorded 7.6 % by Abdel Hamid *et al*. (1984) [110] who investigated Egyptian fenugreek seed. Fenugreek seed contains very important minerals for human body system and has great a beneficial positive effect on the health of human [61]. Fenugreek seed possesses minerals like magnesium, phosphorous, calcium, iron, potassium [16]. Iron is beneficial in the cure of anaemia, phosphorous may effect on bone formation and main metabolic activities of the body, calcium play a critical role in protecting the hardness of the skeleton, and zinc has an antioxidant effect [112,113].

The moisture content was 5.25%. Several reports have shown different amount of moisture content of fenugreek seed, usually moisture content ratio between 5-10 % [16], and the plant materials moisture content could depend on storage conditions, and shelf stability.

Fenugreek seed contained approximately 55.02% carbohydrates. This result was higher than reported by Srinivasan (2006) [16] who determined a value of 48% percent of hydrocarbon content in fenugreek seeds and similar that reported by Vats *et al.* (2003) [56] who determined 55% carbohydrate present in fenugreek seed. Yousif *et al*. (1973) reported the highest amount of carbohydrate present in fenugreek seed by the 60.01%. Carbohydrate fraction of fenugreek seed consists a huge amount of mucilaginous fibre, and galactomannan [4]. Petropoulos (2002) [4] mentioned that the major polysaccharide present in fenugreek seed is galactomannan by approximately half percent of dry seed weight. The present of galactomannan makes fenugreek seed solution like a gel from. Fenugreek seed has a great amount of soluble dietary fibre (SDF), and contains hemicelluloses, mucilage, tannin, saponins, and pectin [15]. SDF helps to decrease the level low density lipoprotein-cholesterol (LDL) in the blood, and fenugreek fibre binds the toxins in the food [8]. According to the Hannan *et al.* (2007) [18] soluble dietary fibre of fenugreek seed effect significantly on type 1 and type 2 diabetes patients, SDF could delay carbohydrate digestion and absorption in the human gut. It has been reported that the crust of the fenugreek is a great source of dietary fibre [9] and could be an effective source of natural antioxidants on functional food applications. As a result, carbohydrate of fenugreek seed has several properties that could attract easily to interest for the further studies, to isolate components and use it on various purpose could provide great opportunities.

3.2. Evaluation of general process of fenugreek seed fractions

In this study, it was obtained four different fractions from the fenugreek seeds as it mentioned previously and completed analyses of their fractions composition and properties. It was obtained four main fractions respectively; Fenugreek ethanol fraction (FEF), fenugreek oil fraction (FOF), fenugreek fermented fraction (FFF), and fenugreek powdered fraction (FPF). Approximately 2 kg of seed were used, 1950 gram ground seed were used for the all process and 1573 gram of total fractions were gathered. Respectively 43.09 gram FEF, 105.08 gram FOF, 238 gram FFF, and 1187 gram FPF were collected and 376.8 gram of seed material was lost during the process. The yield of a general process was 80.6 % g/ 100 g DW. Fractions yield in the process was respectively for the FEF, FOF, FFF, and FPF: 2.19%, 5.38%, 12.19 % and 60.4% g/100 g DW.

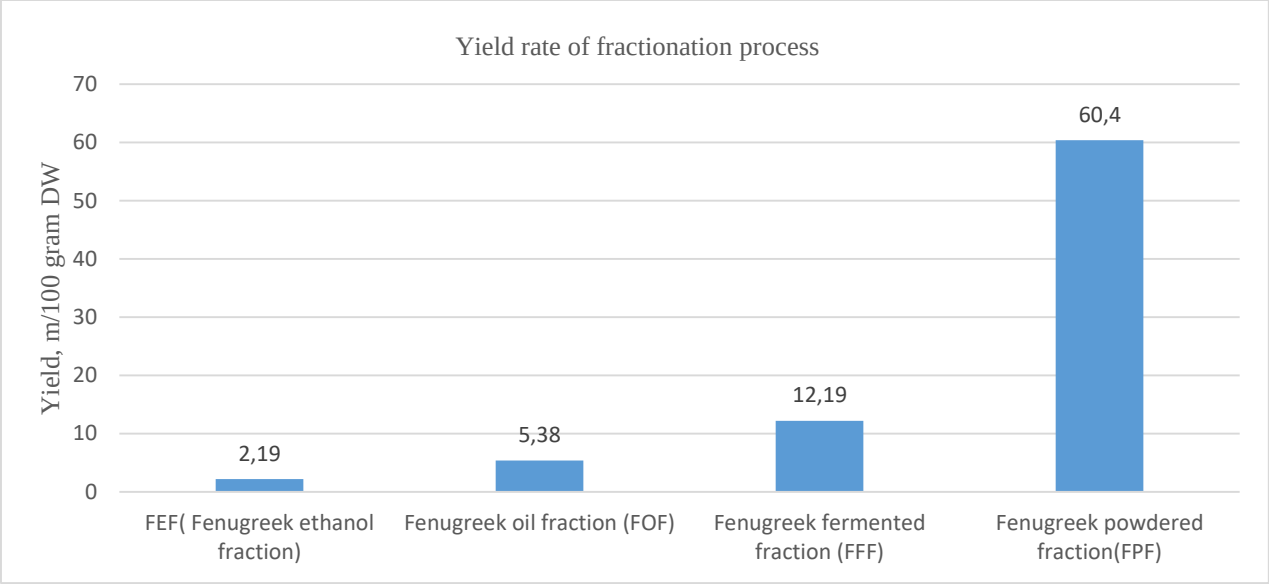


Figure 3.3. Yield rate of general fractionation process obtained from fenugreek seed

Totally 1950 gram dried fenugreek seed was used in ethanol (1/1 v/v) extraction process and 43.09 gram ethanol extract was collected, yield of extract was 2.21% g/100 g DW, 960 gram ethanol used and 650 gram ethanol collected (67.70% yield, g/100 g DW) in ethanol extraction process with the condition described previously. The extract yield (2.21%) was lower than reported by Norziah *et al.* (2015) [35] who found 12.87% extraction yield obtained by oven-dried, and finely powdered fenugreek seed with 1:5 solid/ ethanol solvent ratio. The recovery and yields of biologically active compounds from natural plant origin material can be affected by various factors such as solvent type, extraction terms, and the solid solvent ratio [120]. Thus, this study attempted to extract bioactive compounds used by ethanol due to the reason, ethanolic media prohibit possible bacteria growth for

the safety perspective of the process and less toxic effect of ethanol comparing the other solvents. In addition, fenugreek seed extraction with the hot and cold water causes high viscosity level because of the presence of water soluble gum (galactomannan). [35] Therefore to make a filtration and extraction process could be difficult.

Fenugreek oil, extracted with the SFE-CO_2 method and extraction yield was 5.68 % g/100 gram DW in the SFE-CO_2 process. Extraction results explained in detail in 3.6, paragraph. As a result in this study obtained fenugreek seed oil with the high yield ratio comparing the Renming *et al.* (2012) [115] who reported between 2.70% to 3.76% yield ratio. Fenugreek oil has great economic value and has very important properties for health. According to the Rosell *et al.* (2005) [37] that essential oils from the edible plant as fenugreek with high polyunsaturated fatty acids are recommended on diets for lowering blood cholesterol [37]. The plant-based oils are providing natural sources of n-3 fatty acids and strong antioxidants, for this reason, consumption of fenugreek seed oil can improve human nutrition. At the present time, demand for edible oils is increasing in order to be applied to functional foods, oil extract from fenugreek seed has an antioxidant effect and it may be potential for use as food additives for the human diet [41]. For future, we could say that edible oils from natural plants possess unique properties and it may be used together with new technologies on food.

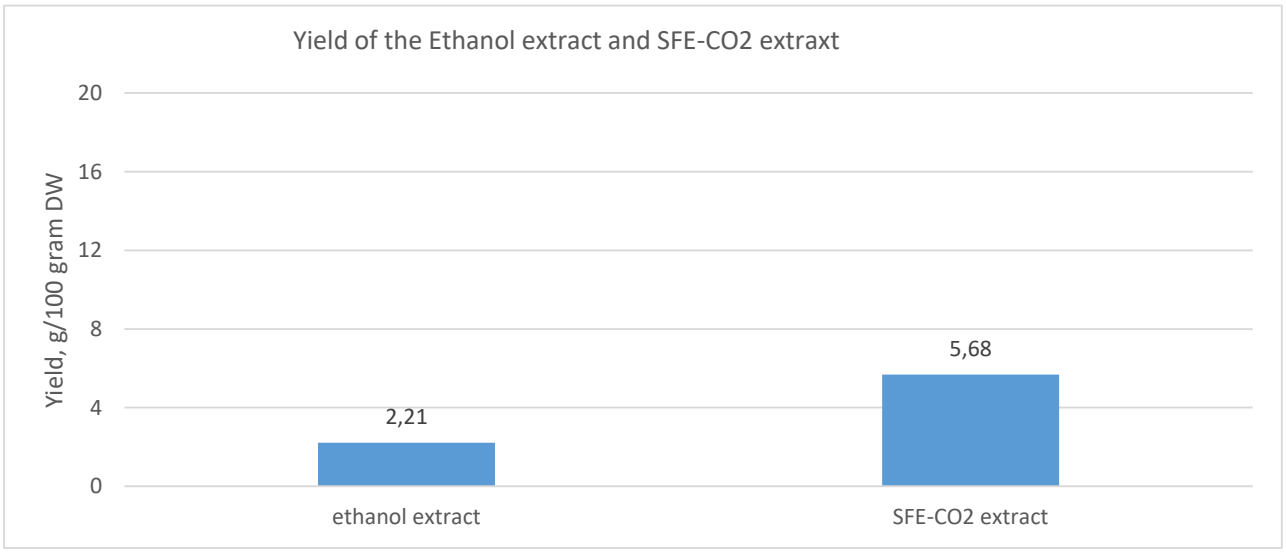


Fig 3.2. Yield rate of Ethanol and SFE-CO_2 extract in process

In fermentation application, approximately 27 kg sample with the (1/15 v/v) ratio fenugreek seed/water used. Totally 238 gram fenugreek fermented fraction (FFF) and 1187 gram fenugreek powder fraction (FPF) obtained from the process with the yield of process respectively; 47.81%, and

74.3%. Those two fractions (FFF, and FPF) possess important bioactive compounds for the health which detailed in below and could be used in food such as supplement for foods, on various functional food application, variously modified fractions of fenugreek seed could be added to foods as baked good for better quality, on meat products to preserve, nutritional supplement or different purposes due to the reason of properties of fractions.

Fenugreek fermented fraction (FFF) possess *Lactobacillus* microorganism, it was detailed on the microbiological result of the study explained in 3.7 paragraph. *Lactobacilli* species are probiotic microorganism and have many health benefits, it promotes digestive tract and immune system health, and it also helps in the production of lactase. This could provide some benefit to those with lactose intolerance [121]. Therefore, the fermented powder could be very useful and have economically great valuable. Furthermore, fenugreek powdered fraction (FPF) that obtained from this study could be considered as an animal feedstock. FPF has a great amount of protein, soluble fibre (carbohydrate) which detailed below. According to the Shah & Mir (2004) [9], fenugreek seed supplemented diet increased cow performance and milk improved milk characteristic without altering milk taste or flavour. Degirmencioglu *et al.* (2016) [122] reported that diet including fenugreek (Trigonella foenum-graecum) seed on Anatolian water buffaloes, and milk production substantially increased by consumption of fenugreek seed supplemented feed. For this reason powdered beneficial fraction could be great source for further studies and economically attracts interest of food industry. As a result, this study aimed to obtain various fractions from fenugreek seed with the fermentation application to be used various purposes.

On the other hand, several studies have been completed to obtain and fractionated compounds such as diosgenin, saponin or fibre from the fenugreek seed. The existence of crude alkaloids and saponins which present in significant amount in fenugreek seed reveal their therapeutic importance. Alkaloids might demonstrate advantageous for recovery injuries, burns, ulcers, and haemorrhoids. Saponin might possess anti-nutritional properties that may reduce the uptake of specific nutrition such as glucose and cholesterol in the gut [137].

Stadtlander (2012) [146] studied to obtain and fractionated crude saponin extract from the fenugreek (*Trigonella foenum-graecum*), tested in vitro and vivo with the different solvent used. It was evaluated that significant biological activity showed saponin fractions used with the together various crude mixture. However, recently there are not many studies have been done on fractionated components from the fenugreek seed by the amount of yield expression, fenugreek seed possesses a

great source of important and beneficial composition that would be separated and use it for several purposes.

3.3. Evaluation of fenugreek ethanol fraction (FEF) and volatile compounds

Natural plant extracts contain various classes of phytochemicals. Those compounds considered to be main biologically active compounds for supplying health benefits. Therefore, total phenolic components, anti-oxidant activity, GC-MS analysis of FEF completed and the result was evaluated below.

Total phenolic content (TPC) of FEF

The content of phenolic compounds was measured and shown in Table 3.8, and it was found that the concentration of phenolic compounds in the fenugreek ethanol fraction was 38.43 mg of Gallic acid equivalents (GAE) in 1 g of extract. Different classes of phenolic always get attracted in the food industry. Phytochemicals have been getting to know to represented antioxidant activity [136]. Norziah *et al.* (2015) [35] investigated on different extract from fenugreek seed, the seed was extracted with ethanol (75%), methanol (75%), water, hot water, and whilst water extract obtained by germinated fenugreek seed. The highest phenolic contents were found 156.3 mg GAE/g in whilst water extract obtained from germinated seed. On the other hand our result obtained by ethanol extracted fenugreek seed was lower than comparing to Norziah *et al.* (2015) [35] reported total phenolic content of ethanol, methanol, water and hot water obtained by fenugreek crude extract respectively; 44.96%, 43.15%, 19.31% , and 25.60%. The phenolic compounds extracted from fenugreek seed depends on highly on the polarity of the extraction solvent [34]. On the other hand germination application on fenugreek seed could reduce sugars and minerals, and decrease polyphenols content of seed [138]. Ethanol usually more productivity on the lower molecular weight of phenols from the extraction process [34].

Table 3.8. Total phenolic content in FEF

Material	**Phenolic compounds (mg/g in GAE) by Folin-Ciocalteu's**
Fenugreek ethanol fraction (FEF)	38.43 ±0.8

Several other studies have been completed, Chan *et al*. (2011) [139] who reported approximately 10 mg GAE/ g of phenolic content from the water extraction of ground fenugreek seed obtained by 1: 20, solids to water ratio with 23% of the yield. Dixit *et al*. (2005) [140] who reported respectively: 64.6 mg GAE/g, and 47.6 mg GAE/g of phenolic content from the germinated fenugreek seed extracted with the water and boiling water. Bukhari *et al*. (2008) [141] who determined total phenolic

content of ground fenugreek seed extracts obtained by ethanol, and methanol. It was found 6.85, and 5.75 mg GAE/ g, respectively. Alzoreky & Nakahara (2001) [142] reported 7.3 mg GAE/g in the fenugreek seed extract obtained by methanol. The total phenolic content obtained from various fenugreek seed extractions depends on the polarity of the solvent type, extraction methods and extraction times [143]. Additionally, phenolic and flavonoid compounds amount could be changed on chemical variety of phytochemicals and complication of composition in herbs sources. As a result proves that in FEF has a good antioxidant effect due to the reason of its phenolic contents.

Anti-oxidant properties of FEF (ABTS$^{\bullet}$+, DPPH$^{\bullet}$ scavenging assay)

Antioxidant activity of fenugreek ethanol fraction (FEF) was evaluated as ABTS (mg TE/g sample; mg TE/g DW), DPPH (mg TE/g sample; mg TE/g DW). Usually, antioxidant activity depends on phytochemical composition and their properties which present in plant material. An anti-oxidant activity of FEF and result was evaluated below.

ABTS$^{\bullet+}$ and DPPH scavenging assay in FEF

The antioxidant activity of FEF was measured and result on ABTS$^{\bullet}$+, DPPH$^{\bullet}$ scavenging assay shown in Table 3.9. In the ABTS scavenging assay, extracts were 32.37±0.26 mg TE/g sample. In the DPPH scavenging assay amount was 7.24±0.39 TE/g sample. The total antioxidant activity was determined inconsistency with the decolonization of ABTS to its radical cation. The using of DPPH and ABTS radical as a substrate is broadly have been used to measure the antioxidant capability of natural herbal plants extracts which indicate higher prevention level is an index of a powerful antioxidant. Various investigations have been completed on fenugreek seed antioxidant activity of fenugreek seed. Vani Pasricha & Rajinder K Gupta (2014) [61] who reported 7.5 IC50 value (mg/ml) on fenugreek seed extract obtained by methanol.

Table 3.9. ABTS$^{\bullet}$+, DPPH$^{\bullet}$ scavenging assay of FEF

Material	**ABTS/ *TEAC mg Trolox/g sample***	**DPPH / *TEAC mg Trolox/g sample***
Fenugreek ethanol fraction (FEF)	32.37±0.26	7.24±0.39

V. Priya *et al.* (2011) [33] who reported 1 mg/ml exhibited 82.05% DPPH scavenging activity tested on ethanolic extract of ground fenugreek seed. Jha & Srivastava (2012) reported 1 mg/ml exhibited 74.16% DPPH scavenging activity tested on whilst methanolic extract of fenugreek seed. Norziah *et al.* (2015) [35] who studied radical scavenging activity of various extracts obtain by used different solvent from the fenugreek seed, and it was reported that DPPH radical scavenging activity

varied concentrations ranged from 0.02 to 2.5 mg/ml, ethanolic fenugreek seed extract was (68.6%) inhibition activity (where the standard antioxidant Vitamin C 0.01 mg/ml and DPPH: 2, 2-Diphenyl-1-picryl-hydrazyl). On the other hand, Chan *et al*. (2011) [139] who reported that approximately 60 times scavenging activity on hot water extracted ground fenugreek seed was lower than comparing to extracts obtained by ascorbic acid. Syeda Birjees Bukhari *et al.* (2008) investigated DPPH activity on fenugreek seed obtained by ethanol and methanol extracted fenugreek seed, and ethanol extracted fenugreek seed was showed highest scavenging activity than methanol extracted fenugreek seed. The results indicate that phenolics amount of extracts and type of different solvents used on extraction have a significant effect on free radical scavenging. According to the Yildirim *et al.* (2001) [144] for the samples of plant material, the antioxidant activity is related with their power of bioactive compounds. Mashkor (2014) [159] reported that free radical scavenging activity of fenugreek seed decreases in parallel with increases in the organic solvent concentration.

Volatile compounds of FEF obtained by GC-MS

Three main fractions: fenugreek ethanol fraction (FEF), fenugreek fermented fraction (FFF) and, fenugreek powdered fraction (FPF) was subjected GC/ MS analysis. Twenty compounds were identified in fenugreek ethanol fraction and obtained by GC-MS analysis. The main principles with their retention time (RT) and molecular concentration presented in Table 3.3 and Figure 3.8. In GC-MS analysis of seed extract some volatile compounds in composition repeated, that means some of them are stereo isomers and have similar mass and atoms but they are different conformation ant has slightly different names.

It was observed that the ethanol extract of fenugreek seed (FEF) contained components which is more than %1, in high to low concentrations as follows; Linolenate <methyl-> (34.04 %), Hexadec-6-enoic acid <16-hydroxy-> omega lactone (19.41%), Hexadecanoic acid <n-> (15.72%), Hexadecanoic acid <n-> (6.45%), Linoleate <methyl-> (5.37%), Neophytadiene (4.33%), Epicubenol (2.62%), Palmitate <ethyl-> (1.61%), Heneicosane <n-> (1.35%) and the Nonadecane <n-> (1.31%). Linolenate <methyl-> is a derivative of Lineolic Acids which possess antioxidant properties. It is used as a flavoring ingredient and it might be used as a model compound in oxidation test to estimate the opponent-peroxidation activity of ellagitannins, fullerenes and other natural products. The application of methyl linolenate is the oxidation of compound in organic solution and phosphatidylcholine (PC) in liposomal membranes induced by an azo initiator has often been used as a model for lipid peroxidation in vivo [147]. Neophytadiene is an enzyme inhibitor, blocking and enzyme's activity could kill a pathogen, and many medicines are enzyme inhibitor, besides it could be used in pesticides

[148]. Hexadecanoic acid<n-> is the saturated fatty acid and naturally occurs in plant materials. In accordance with a Chen (2010) [149] who was investigating plant extract and reported that palmitic acid which presents in the natural plant has antioxidant properties and it can help prevent several diseases which were investigated in rats.

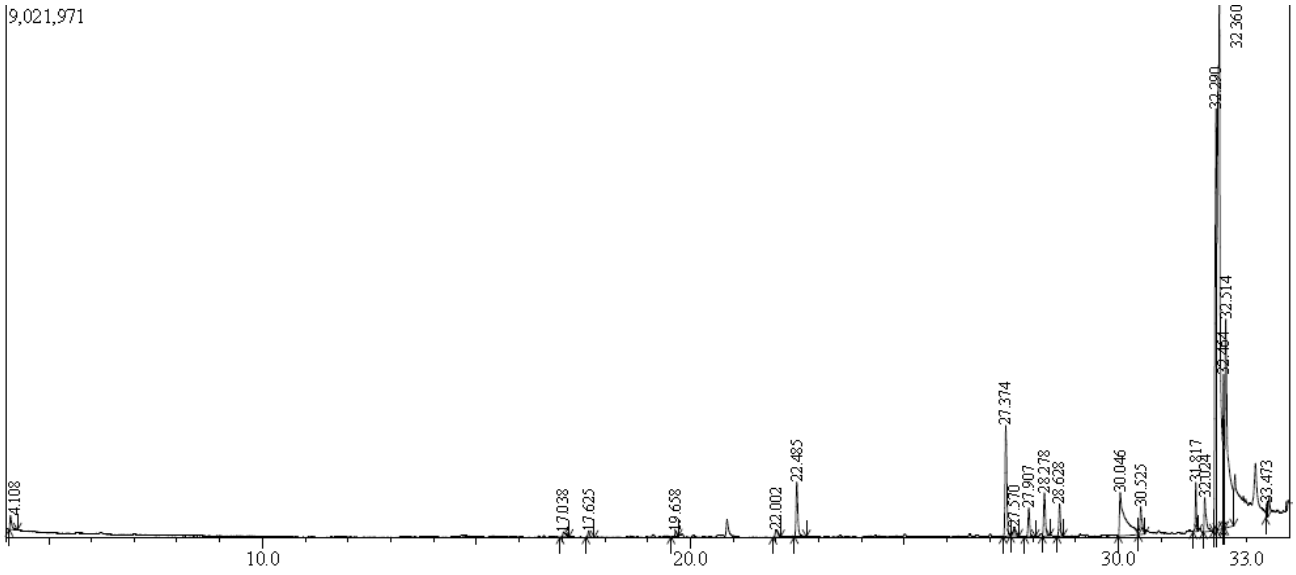


Figure 3.8. Screening result of FEF by GC-MS

Similar studies have been completed on investigating of fenugreek seed extract. V. Priya & Vijayalakshmi (2011) [33] who analyzed fenugreek seed, extracted with ethanol, and identified compounds by the following name; a-D-Glucopyranoside, methyl (74.5%), 3-O-Methyl-d-glucose (16.11%) 2- Propen-1-amine, N- ethyl- (3.43%), Aziridine, 1,2,3-trimethyl-, trans- (2.41%). Those compounds possesses such as antimicrobial, antioxidant, anti-cancer, and Hypocholesterolemic properties [33]. Pasricha & Gupta (2014) [61] investigated on fenugreek seed and leaves, and who reported phytochemicals which are main for the regulation function such as blood pressure, lipid levels, immune response ,and inflammation responses.

As a result, fenugreek extract has several important compounds such as Linolenate <methyl->, Hexadecanoic acid<n->, Hexadec-6-enoic acid <16-hydroxy-> omega lactone, which possess antioxidant properties and it proves that fenugreek ethanol fraction possess useful composition, for the further work, FEF might be used for various purposes on food or pharmaceutical industry.

Table 3.3. Compounds of FEF identified by GC-MS analyse

Peak	R.time	Area	Area%	Height%	Name of compound
1	4.108	662387	0.62	0.78	Nonane<n->
2	17.038	383889	0.36	0.28	Decanonate<ethyl>
3	17.625	290092	0.27	0.35	Caryophyllene<E->
4	19.658	249006	0.23	0.31	Cadinene<gamma->
5	22.002	504241	0.47	0.38	Dodecanoate<ethyl->
6	22.485	2790798	2.62	3.04	Epicubenol
7	27.374	4606512	4.33	6.18	Neophytadiene
8	27.570	475042	0.45	0.45	Phytone
9	27.907	1343668	1.26	1.60	Neophytadiene
10	28.278	2057349	1.93	2.41	Neophytadiene
11	28.628	1392753	1.31	1.80	Nonadecane<n->
12	30.046	6860549	6.45	2.40	Hexadecanoic acid<n->
13	30.525	1717515	1.61	1.54	Palmitate<ethly->
14	31.817	1431454	1.35	2.73	Heneicosane<n->
15	32.024	1857071	1.75	1.89	Neophytadiene
16	32.290	20649473	19.41	23.53	Hexadec-6-enoic<16-hydroxy->omegal
17	32.360	36205327	34.04	29.32	Linolenate<methly->
18	32.464	5715059	5.37	8.59	Linolenate<methly->
19	32.514	16722237	15.72	11.64	Hexadecanoic acid<n->
20	33.473	449589	0.42	0.78	Tricosane<n->

3.4. Evaluation of Fenugreek oil fraction (FOF)

The physicochemical of FOF was investigated. The colour of oil at the room temperature was brownish yellow. The peroxide and iodine value was expressed g/100 g oil and result is shown in table 3.6. Peroxide value was 0.414 ±0.12 g/ 100 g of oil. This result was lower than Ling-Biao *et al,* (2016) [145] who reported 0.627 ± 0.033 meq. O2/kg oil. The peroxide value of the oil was lower due to the reason during the SFE-CO_2 extraction or different process which oil exposure under different conditions. During the storage and temperature might effect on the peroxide result. Iodine value was 144.54 ±0.132 g/ 100 g of oil. This result was similar according to the Ling-Biao *et al,* (2016) [145] who reported 148.564 ± 2.025 (g/100 g oil). Iodine value of the oil depends on saturated fatty acids

(SFA). High iodine value shows that in fat possess low content of saturated fatty acids. Oxidation of lipids is the main parameter that evaluate oil as edible.

Table 3.6. Physicochemical characteristics of FOF

Properties	**Values**
Iodine value (g/100 g oil)	144.54 ±0.13
Peroxide value (g/100 g oil)	0.414 ±0.12

In general, fenugreek seed oil has high antimicrobial activity according to the Sulieman (2008) [41] and it is acceptable edible oil for consuming, recommended to improve the shelf life of food and could be promising for the using on new food application. Ling-Biao *et al.* (2016) [145] who investigated physicochemical characteristic of fenugreek oil extracted by subcritical butane extraction, and found refractive index of 1.479, this result was similar to corn oil (1.473), and soybean oil (1.477), refractive index is one of the quality index of edible oil which highly depends on unsaturated fatty acids. Fenugreek seed has high amount of unsaturated fatty acids such as oleic acid, linoleic acid, and Linolenic acid. The acid value, density of the oil, saponification value of the oil, and unsaponifiable matter found to be respectively: 6.413 mg/g oil, 190.277 mg KOH/g of oil, and 3.790% [145]. That value shows that fenugreek seed oil has quite good physicochemical characteristics for the consuming.

Fatty acid compositions and oxidation properties FOF

Non-polar constituents isolated by supercritical carbon dioxide extraction (SFE-CO_2)

Non-polar (oil) constituents from fenugreek seed was extracted with supercritical carbon dioxide extraction (SFE-CO_2) method by using approximate optimum extraction conditions according to the Renming *et al.,* (2012) [115] with 25 MPa , 40 °C, and 200 minute extraction time. The oil obtained with SFE-CO_2 extraction process chart and extraction parameters shown in figure 3.4. Non-polar fraction yields was 5.68%, and it was expressed as g/100 g DW of plant material. Yield result was higher than reported by Renming *et al.,* (2012) [115] who reported the yield of SC-CO_2 extracted fenugreek seed and ranged between 2.70 % to 3.76%. Similar studies have been completed, Stamenic *et al.,* (2014) [129] reported reported varying yields for Greek oregano at different SFE-CO_2 conditions: 0.74 g/100 g (30 MPa, 40°C, CO_2 flow rate 2.7l/min), 1.02 g/100 g (10 MPa, 40°C, CO_2 flow rate 4.5l/min), 1.5 g/100 g (30 MPa, 100°C).

This study was focused on new extraction method such a SC-CO2 to obtain huge amount non-polar constituents from fenugreek seed due to the reason; result was indicated that huge amount of samples could be extracted in short time and non-toxic reside left after extraction compared to the conventional extraction method [116]. For the SC-CO_2 extraction method, three main parameters to influence on extraction yield respectively; pressure of extraction process, extraction time and extraction temperature [117]. Supercritical carbon dioxide extraction (SC-CO_2) has several good properties, with the inclusion of non-toxic, cost efficient, and it could easily eliminate from extracts, those properties makes very attractive to use SC-CO_2 method instead of classic methods.

Fatty acid composition obtained by GC-MS

Fenugreek oil has been isolated from the fenugreek seeds through SC-CO_2. The oil content in examined fenugreek seed ranged from very less amount (0.001%) to 39.022%. Fatty acids composition were analysed by typical gas chromatograph and determined fatty acid shown in Table 3.2. Six different fatty acids were identified which amount was higher than 1% and the fatty acids were identified in high to low concentrations as follows: Linoleic acid (39.022 ± 0.30), Heneicosanoic acid (26.018± 0.17), Palmitic acid (11.009 ±1.30), Stearic acid (4.273±0.32), and Arachidic acid (1.116± 0.27). Linoleic acid was the highest amount and comparing to the Shahat (1947) [123] who examined Egyptian fenugreek seed and Badami & Kalburgi (1969) [125] who investigated Indian fenugreek and determined linoleic acid amount respectively; 13.8% and 13%, observed significant differences. However linoleic acid result was nearly comparing to the Sulieman (2008) [36] who found linoleic acid amount 43.2 % while investigating Sudan fenugreek seed and Rathore *et al.,* (2016) reported 43.11 % linoleic acid. Those result indicated that range of fatty acid composition could change from different origin and varieties of seeds. Hilditch & Williams (1964) [125] mentioned that different temperature and atmosphere are main factors for different account of linolenic acid. Because of the fatty acid profiles, fenugreek seed oil gets into drying oil category [36]. When the drying oil oxidizes, it could form a rigid and flexible film by exposed to the air.

In the study, result was in agreement with reported common fatty acids presence in fenugreek, except Heneicosanoic acid. Approximately 26 % Heneicosanoic acid was determinate in our analyses. At least Shailendra Nath Saxena *et al.,* (2016) [126] reported very less amount of Heneicosanoic acid (0.001%). Change in amount of the fatty acid might be effect by different genotype, growth conditions or various analytical system applied to the determination of composition. At the end because of the fatty acid composition, fenugreek oil possesses potent anti- diabetic, lowering the risk of heart

diseases, and with high polyunsaturated fatty acids, fenugreek seed oil may help to lower blood cholesterol [37].

Table 3.2. Fatty acid present in fenugreek seed oil

Fatty acids	**Retention Time (min)**	**Peak area %**
Palmitic acid	40.73	11.009 ±1.30
Stearic acid	44.49	4.273±0.32
Oleic acid	45.67	14.167±0.78
Linoleic acid	47.53	39.022± 0.30
Arachidic acid	47.95	1.116± 0.27
Heneicosanoic acid	49.53	26.018± 0.17
Myristic acid methyl ester	35.52	0.198 ± 0.68
Pentodecanoic acid methyl ester	38.65	0.153 ±0.55
Cis-10- Pentadecenoic acid methyl e.	39.8	0.215 ±0.48
Heptadecanoic acid methyl ester	42.55	0.307 ±0.39
Behenic acid methyl ester	51.35	0.458 ±0.32

Oil stability in Oxi-press method

Fenugreek oil extracted from the fenugreek seeds through SC-CO_2 antioxidant activity measured by Oxi-press method. It was used as well rapeseed oil for comparing to fenugreek seed oil. Oxi-press method was based on accelerated oxidation measurements. The measurement was lasts until secondary oxidation products are observed. The result is shown in Figure 3.5, for the control sample it was used rapeseed oil. Induction period was for the fenugreek seed oil and the rapeseed oil was respectively; 2.2 and 3.3 hours. That result indicated that rapeseed oil has better antioxidant stability than fenugreek oil. However, oil stability could change according to the temperature and storage conditions. Fenugreek seed oil kept in freezer and quality of the oil could be decreased during the storage or espoused air for a while. Trojakova & Coworkers (2000) who investigated oil stability on sage and rosemary extracts in refined rapeseed and sunflower oil. Extracts were prepared by soaking plant materials in hexane, acetone and ethanol for 24 h at room temperature and ambient pressure in the dark. In all oils, there were used 0.05% of different extracts and Oxi-press carried out at 100°C. With sage acetone, ethanol, and hexane extracts induction periods in rapeseed oil were 11.26h, 11.26h, 11.70h respectively (control sample – 8.80h). In sunflower oil – 8.3h, 8.03h, 8.77h, respectively (control sample – 6.64h). With same solvents rosemary extracts in rapeseed oil induction periods were as follows: with acetone – 12.50h, with ethanol – 12.06h. In sunflower oil: with acetone – 9.56h, with ethanol – 8.63h. Some studies have been completed on fenugreek seed oil. Ling-Biao *et al,* (2016)

[145] who reported the induction time for the fenugreek seed oil and result was 2.850 h at 120°C, this result was quite similar which founded in this study. That result shows that induction period of the fenugreek seed was lower than other edible oils such as olive oil, and rapeseed oil, and that results might be changed due to the reason of amount of the polyunsaturated fatty acid (PUFA) composition. The fenugreek seed oil contains a higher amount of PUFA comparing to the olive oil [146].

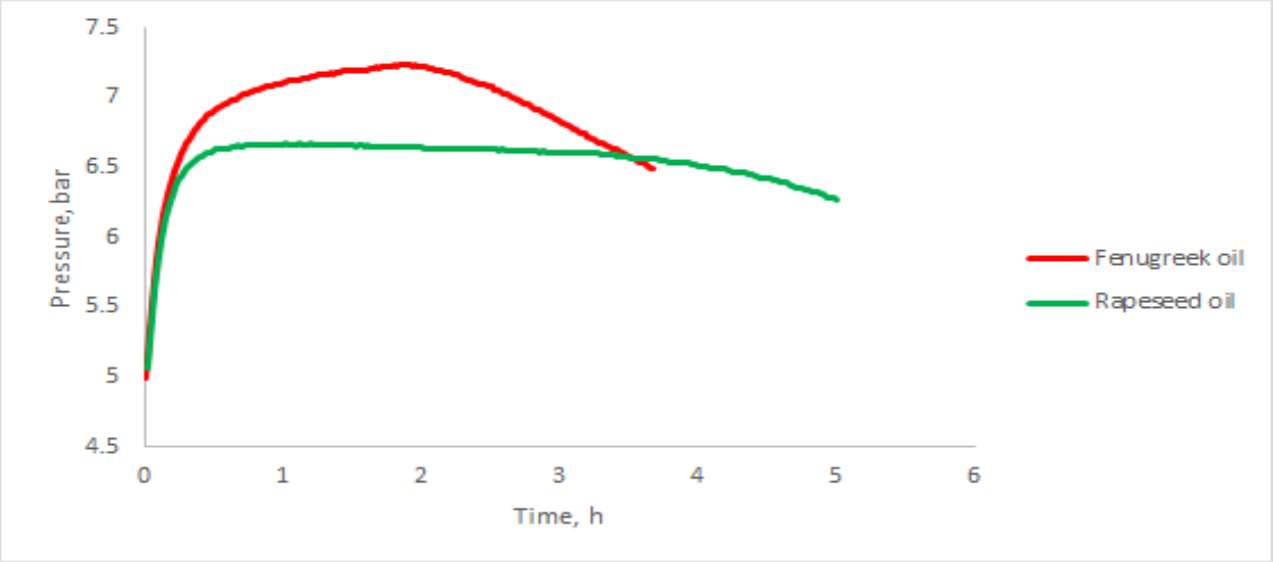


Fig 3.5. Oxidative oil stability of fenugreek and rapeseed oil

3.5. Evaluation of Fenugreek fermented fraction (FFF)

The FEF evaluated by their microbiological properties and volatile compounds were determined with the GC-MS analyses. The result and assessments was detailed below. The microbiological evaluation were the most important for FFF due to the reason of probiotic microorganism growth rate of the FFF.

Microbiological analysis of fermented fenugreek seed fraction

Fermentation process improves and positively effect on product quality and nutritional value. Therefore in this study aimed to apply fermentation process on fermented fenugreek fraction (FFF) with *Lactobacillus casei* microorganism, and observed growth of the microorganism number of the final product. The microbiological result was observed according to the parameter of TTA (total titratable acidity value), pH value and microbiological growth of *Lactobacillus casei*. Properties of the fermented powder determined during 24, 48 and 72 hours. Microbiological growth observed at the beginning of the fermentation and after 120 hours. The result of total titratable acidity shown in figure 3.6, ranged between 5. 54 to 7.1, during the 24h to 72 hours. The TTA value increased during the

fermentation time. This result showed that during the fermentation acidification of fermentation media increased. The pH values during the fermentation reduced and ranged from 6.18 to 5.32 after 72 hours, pH value changes shown in figure 3.4. The pH value and acidification efficiency of the *Lactobacillus* microorganism demonstrated that most of the advantageous properties related to the fermented products. The formation of the acids depends on parameters such as fermentation temperature and time, the regulation of acidification changes with a number of fermentable carbohydrates [126].

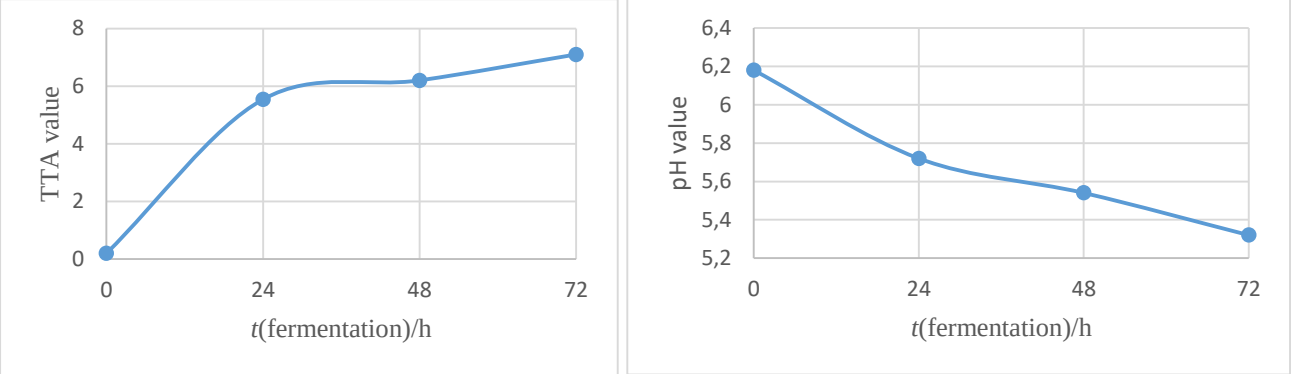


Fig 3.4. The values of total titratable acidity and pH of FFF during the fermentation

The microbiological analysis and counting of microorganism amount of fermented fenugreek seed samples made under laboratory conditions, Lactobacillus casei counted the at the beginning and after 5 days of fermentation, ranged 1.5×10^5 to 20×10^7 (CFU/g). The result indicated that with the appropriate conditions and terms, the number of microorganisms increased. The Figure 3.7 shown that increased of *L. casei* according to the log CFU$\times10^6$/g. Productivity and yields are important for the fermentation process. The temperature of fermentation, pH of the medium, properties of the solvent, incubation times and several factors are effect on fermentation process [44]. As a result, analyses fermentation of the fenugreek seed fraction was successfully completed. In this study was determined that growth conditions of fenugreek seed could be respectively; 37 °C, 48 hours, and with the water solvent as similar comparing to the Hussain *et al.* (2016) [43] who reported for optimum growth conditions and appropriate microorganism for fermentation on several herbal formulation respectively; Ginseng seed (*L. gasseri, Pediococcus pentosaceus, B. subtilis*, MRS broth, water solvent, 30 °C, 24 h), Ginkgo biloba leaves (*Aspergillus niger*, 10 g of solid medium and 16 mL of nutritive salt, 30–40 °C for 6 days.

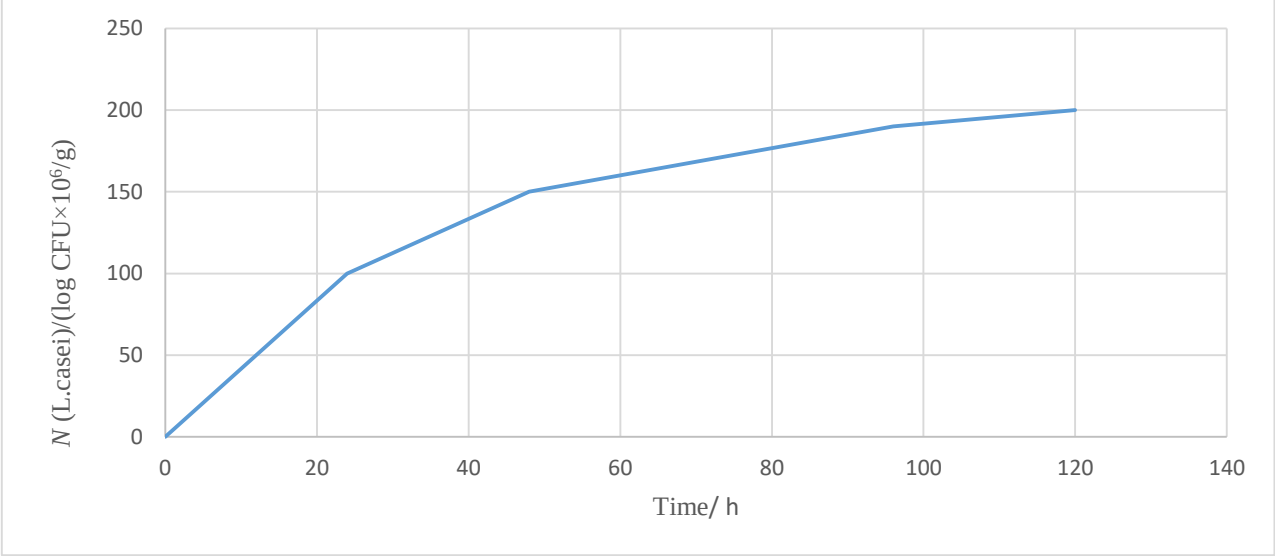


Fig 3.7. *L. casei* propagation in the prepared fermented products

However in this study focused on microbiological growth on natural plant material, that fermentation process improves biological features of plants and herbs. In more detail, fermentation effects on complex substances of plant compounds and changes them into compatible substances, this process associated with several biochemical changes, it related ratio of different nutritive value, bioactivity and digestibility [43]. According to the Lee Kim, & Kim (2015) [127] who investigated fermented ginseng seed and the results showed that fermented ginseng seed was higher antioxidant activity and total phenolic content, comparing with unfermented seeds. On the other hand, fermentation process that increases the phenolic content of plant materials [39]. Dordević & Dimitrijevic (2010) [111] who reported fermentation enhance the antioxidant effect of several plants and plants products by increase their DPPH radical scavenging activity on free radicals. On the plant cell walls, structural breakdown is leading to deliver different antioxidant substances which capture free radicals through with fermentation process. Fan, Zhang & Li (2009) [128] investigated soybean product, it was observed that several changes occur on macromolecular properties and antioxidant effect of products by increasing with the presence of fermentation-mediated substrates. Studies proved that relationship between polyphenolic compounds of plants extracted materials and antioxidant effects are closely related.

At the end fermented fenugreek fraction was finely powdered, it may be used for the further studies or could be applied on different food as a supplement and increase their nutritional quality, and might provide great economically benefits for the food industry.

Volatile compounds of FFF analyses

GC-MS results revealed on fenugreek fermented powder of modified fenugreek seed fraction and eleven compounds were identified. Name of compounds present in powder were; Totaldehyde<para-> (60.9%), Guaicol <4-ethy> (14.66%), Nonanal<n-> (6.59%), Epicubenol (3.87%), Nonanol (3.43%), Decanal<n-> (2.39%), Eugenol (2.24%), Hexadecanoate<methyl-> (1.72%), Guaiacol (1.60%), Tetrahydrofurfuryl acetate (1.43%), Tridecanol<n-> (1.18%) respectively. Comparing to the powdered fractions (FPF), on the fermented powder five different compounds determined. Those compounds are; Tolualdehyde <para->, Guaiacol, Guaicol <4-ethy>, Eugenol, and Hexadecanoate<methyl->.

Table 3.5. Compounds of FFF identified by GC-MS analyse

Peak	R.time	Area	Area%	Height%	Name of compound
1	8.799	226398	1.60	1.28	Guaiacol
2	8.981	931966	6.59	11.78	Nonanal<n->
3	10.914	485382	3.43	3.84	Nonanol
4	11.809	338365	2.39	5.02	Decanal<n->
5	12.441	8618320	60.90	46.07	Tolualdehyde <para->
6	13.960	2074247	14.66	15.48	Guaicol<4-ethy>
7	16.160	317335	2.24	1.97	Eugenol
8	21.933	202005	1.43	2.17	Tetrahydrofurfuryl acetate
9	22.475	547876	3.87	7.90	Epicubenol
10	23.883	166923	1.18	1.57	Tridecanol<n->
11	29.225	243777	1.72	2.92	Hexadecanoate<methyl->

Totaldehyde<para-> is an aromatic aldehyde, and it is used in the preparation of (E)-3-(4-methyl-phen-yl)-1-(1, 3-thia-zol-2-yl) prop-2-en-1-one, and it used in the preparation of (Z)-trisubstituted allylic alcohols [150]. Compound is commercially available. Guaicol <4-ethy> is a phenolic compound, it is cultured in wine and beer by the yeasts, and compound can effect organoleptic properties of the wine [151]. The compound used as flavouring ingredients and commercially available. Nonanal<n-> is an alkyl aldehyde, it is colourless, oily liquid form, and nonanal is a component used on perfume production. Generally it occurs in several natural oils, and produced commercially [152]. There is interesting information that nonanal has been recognized as a compound which attracts mosquitos [153]. Guaiacol is an organic yellowish aromatic oil compound derived from guaiacum. The compound participates to the flavour of numerous compounds such as roasted coffee [154]. Guaiacol has been improved for extraction and used commercially.

Hexadecanoate<methyl-> (Methy Palmitate) is a fatty acid compound which belongs to class of fatty acid methyl esters. Methly Palmitate has anti-inflammatory and anti-fibrotic effect, and it prohibits lung inflammation and fibrosis in rats reported by Ebtehal El-Demerdash (2011) [155].

Other compounds presented in FFF generally organic compounds that used in flavouring agents. Consequently, there is volatile aromatic compounds present in fermented fraction of fenugreek, and some of the components possess anti-oxidant, anti-inflammatory, anti-fibrotic effect besides microorganism growth on the fractions proves that fermented fenugreek fractions might be used on various purposes, it could be used such as supplement for food to improve nutrition quality or natural medicine components for the pharmaceutical studies.

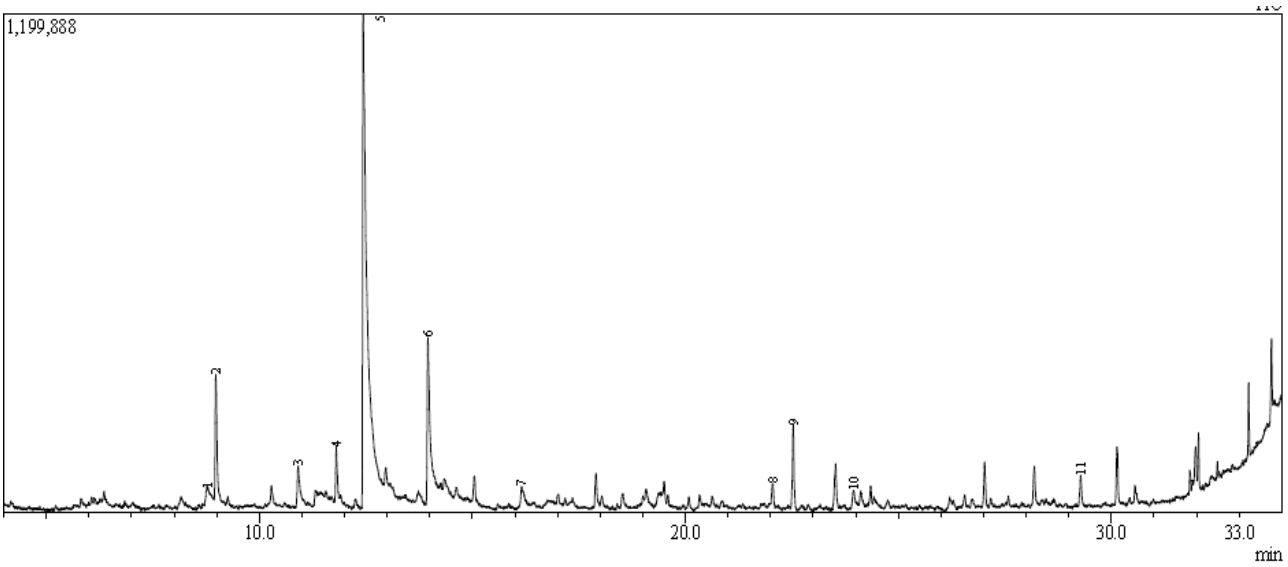


Fig. 3.10. Screening result of FFF by GC-MS

3.6. Chemical composition of fenugreek powdered fraction (FPF) and volatile compounds

The proximate protein, fat, mineral, moisture and carbohydrate content for fenugreek powdered fraction (FPF) and presented in Table 3.7. The amount of the protein, fat, mineral and moisture content was similar as fenugreek seed. The protein content of fenugreek powdered fraction was 30.52 %. This result was quite similar compared to the fenugreek seed which was founded (29.11%) and protein content might change because of the different process application on fractions. Fat content was 2.11% for the powdered fenugreek fractions. This result was less than fenugreek seed fat content which was done at the beginning of the process due to the reason, fenugreek oil was extracted during the SFE-CO_2 application. Moisture content was lower than 1%, this result was lower comparing the fenugreek seed which was done at the beginning, and the fraction was powdered form after the lyophilization process. Freeze drying process evaporated huge amount water on the fraction. Fractions were finely

dried due to the reason, microorganism and contamination rate lower with the less water activity could be useful in powder form, storage and transportation could be easy, and could be used on various food application as a supplement. Mineral content was 3.4% for the FPF. Mineral content might be changed during the process because of the temperature changes. Comparing to the fenugreek seed mineral content which was done, this result was lower. During the process applied on fenugreek seed, mineral, protein, moisture, fat, and the carbohydrate amount has changed. Various process application effected on chemical composition of samples.

Table 3.7. Chemical composition of fenugreek powdered fraction

Index	**Amount, %**
Protein content	30.52 ± 0.65
Fat content	2.11 ± 0.49
Mineral/ ash content	3.40 ± 0.02
Moisture content	0.23 ± 0.12
Carbohydrate	63.74 ±0.10

Carbohydrate amount was 63.74%. This result was higher than comparing to the fenugreek seed which was determined 44.98 %. As previously described that carbohydrate from fenugreek seed such as soluble dietary (SDF), and mucilaginous fibre has several health benefits and several reports indicated that SDF decreases cholesterol level in the bloodstream. As a result, the chemical composition of the powdered fenugreek seed fraction (FPF) seed possess high amount of protein, carbonhydrate and it was identified several biologically active compounds by GC-MS analysis. Car Besides economically could have great benefits. FPF has a big amount of protein and carbonhydrate it could be considered to use as animal and feedstock for the cows and farm industry [122].

Volatile compounds of FPF analyses

The samples of beneficial powder fractions GC-MS analyses was completed. It was observed from the Figure 3.9, and Table 3.4 that 9 compounds were identified. Powdered fractions contained components which is more than 3%, in high to low concentration as follows; Nonanal<n-> (36.90%), Decanal<n-> (14.55%), Epicubenol (12.42%), Heptadecane<n-> (10.28), Tetrahydrofuryl acetate (5.34%), Nerylacetone (4.92%), Octanal<n->(4.90%), and Tridecanal (3.28%). The highest amount of compound was Nonanal<n-> (36.90%). It was used as flavoring agent in the food industry, nonanal is colourless, oily liquid, and component of perfumes [130]. Additionally, Nonanal is a compound that could attract mosquitoes [131]. Decanal which presents 14.55% is an organic compound occurs

naturally and it used flavoring agent, important component of buckwheat odor. In additionaly, Decanal is the main component of coriander herb (*Coriandrum sativum*) essential oil by the 17% of herbs [132]. Tetrahydrofurfuryl acetate which presented approximetly 5% in sample. It is organic compounds used for flavoring agents like honey, maple, or bread-like [133]. Tridecanal is a volatile flavor component which mainly found in citrus and cucumber oil, it has antioxidant properties and belongs to the family of fatty aldehydes [134].

Table 3.4. Compounds of FPF identified by GC-MS analyse

Peak	R.time	Area	Area%	Height%	Name of compound
1	6.352	246969	4.90	3.79	Octanal<n->
2	8.977	1858600	36.90	38.48	Nonanal<n->
3	11.808	732932	14.55	12.53	Decanal<n->
4	15.049	373278	7.41	8.74	N/D
5	18.520	247716	4.92	3.53	Nerylacetone
6	22.052	269049	5.34	5.08	Tetrahydrofuryl acetate
7	22.531	625469	12.42	13.30	Epicubenol
8	24.353	517959	10.28	12.06	Heptadecane<n->
9	24.769	165396	3.28	2.49	Tridecanal

The compound which identified on FPF discussed. Nonanal <n-> was presented highest amount, the compound also identified on FFF. As described previously Nonanal<n-> is an alkyl aldehyde, it is colourless, oily liquid form, it is a component used in perfume production, usually occurs in several natural oils, and it is commercially available [152]. Decanal<n-> is a simple carbon aldehyde, it occurs naturally in plant material, it is used flavouring agent and presented in perfumes. It is important component in citrus throughout with the several other components [156]. Octanal<n-> is an aldehyde and usually presented in citrus, it has odour as fruit-like, and it is colourless. Octanal usually has been used in the food industry as a flavour, and commercially available for the perfumery industry [157]. Tetrahydrofuryl acetate which presented approximately 5 % of FPF is an organic compound (heterocyclic ester) which has been used for food flavouring, also it reputed as maple, honey or bread-like flavour. Those compounds generally recognized as safe in the USA. The compound which presented in FPF is generally natural aromatic compound and used as flavouring agent in food or material for the fragrance in perfume industry. There is no toxic information of the compounds which identified by GC-MS obtained with head-space technology. Generally evaluated that fenugreek powdered fraction is present high amount of protein, carbohydrate, and it has several

biologically active compounds, thereupon FPF could be considering as feed for the animal or great opportunities to investigate more detailed for the future studies.

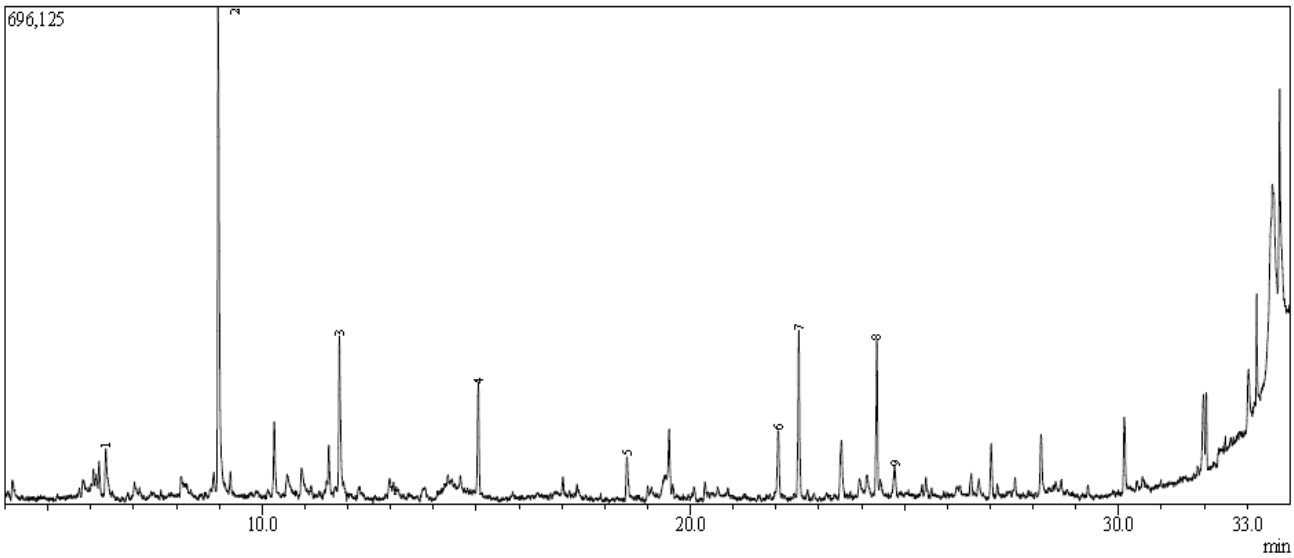


Figure 3.9. Screening result of FPF by GC-MS

CONCLUSIONS

1. Evaluation of chemical composition of fenugreek seed was completed and protein, carbohydrate, fat, mineral, and moisture content estimated with the appropriate method used. Protein content was 29. 11%, fat content 6. 57%, mineral content 4.05 and carbohydrate content 55. 02%. As a result, fenugreek seed is a good source of protein, carbohydrate, and minerals.
2. It was obtained from dry fenugreek seed four fractions: ethanol fraction (FEF), oil fraction (FOF), fermented fraction (FFF) and powdered fraction (FPF). Totally 1950 gram ground seed was used, 1573 gram of fractions was collected, and 376.8 gram seed material was lost during the process. The yield of the fractionation process was calculated 80.6% g/ 100 g DW (from dry weight of fenugreek seed). Fractionation yield in the process was respectively for the FEF, FOF, FFF, and FPF: 2.19%, 5.38%, 12.19% and 60.4% g/100 g DW (expressed dry weight of the fractions)
3. Evaluation of antioxidant properties of fenugreek ethanol fraction (FEF) by total phenolic content (TPC), ABTS, and DPPH assay revealed that FEF possessed good antioxidant properties. TPC result was 38.43 mg /g in (GAE), ABTS scavenging assay was 32.37±0.26 mg TE/g sample, and the DPPH scavenging assay amount was 7.24±0.39 TE/g sample. Results indicated that natural plant material is possessed antioxidant activity and depends on extraction techniques, parameters and different solvents used on extraction. GC-MS analyse completed by the head space technology, it was presented phenolic, and biologically active compounds such as Linolenate <methyl->, Hexadecanoic acid<n->, Hexadec-6-enoic acid which possess anti-oxidant, anti-inflammatory properties.
4. Fatty acid composition, oxidation properties evaluated on FOF. To obtain oil from the fenugreek seed SFE-CO_2 method was applied, and fatty acid composition of FOF revealed by GC-MS analysis. It was found six different fatty acid: Linoleic acid (39.02 %), Heneicosanoic acid (26.01 %), Palmitic acid (11.00 %), Stearic acid (4.27 %), and Arachidic acid (1.116. Oxidative oil stability revealed by the Oxi-press method and induction period was 2.2 h. This result is quite low compared to the other natural oil obtained by plant material due to the reason that amount of the PUFA. FOF contained a high amount of PUFA.
5. It was applied fermentation with used *Lactobacillus casei* by appropriate conditions on FFF. The amount of lactobacillus casei determined at the beginning and after 5 days of fermentation, it was ranged 1.5×10^5 to 20×10^7 (CFU/g). The determination indicated that with the appropriate conditions, the number of microorganisms increased on FEF. Volatile compound of FFF analysed by GC-MS and result revealed that eleven compounds identified. Hereby, there are volatile

aromatic compounds present in fermented fraction of fenugreek, and some of the components possess anti-oxidant, anti-inflammatory, anti-fibrotic effect. As a result, FFF possessed beneficial microorganism and biologically active compound, and it could be used as a supplement on foods.

6. Evaluation of chemical composition and volatile compound of FPF was identified. FPF presented 30.52 % protein, 2.11 % fat, 3.40 %, 0.23% moisture, and 63.74% of carbohydrate. Volatile compounds identified and nine compound was identified. Those compounds were generally used as flavouring agent in foods. The results indicated that FPF possesses a great source of protein and carbohydrate. Because of the powdered form, and contained a high amount of hydrocarbon, FPF could be used on animal diet or feedstock.

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DEDICATION

This thesis is dedicated to my parents who have supported me all the way since the beginning of my studies. Also, this thesis is dedicated to my friend Laurynas who has been a great source of motivation and inspiration. Finally, this thesis is dedicated to all those who believe in the richness of learning.

ACKNOWLEDGEMENT

I would like to take this opportunity to express my heartfelt gratitude to several key individuals who were instrumental to my success of completing this thesis.

I would like to express my gratitude to my supervisor Associate Professor dr. Jonas Damašius for the useful comments, remarks and engagement through the learning process of this master thesis. Furthermore I would like to thank dr. Dalia Cizeikiene and Rita Kazaneviciene for introducing me to the topic as well for the support on the way. Also, I like to thank the participants in my survey, who have willingly shared their precious time during the process of interviewing. I would like to thank my loved ones, who have supported me throughout entire process, both by keeping me harmonious and helping me putting pieces together. I will be grateful forever for your love.

I would also like to acknowledge Associate Professor dr. Ina Jasutienė as the reviewer of this thesis, and I am gratefully indebted to her very valuable comments on this thesis.

Finally, I must express my very profound gratitude to my parents for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

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