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Biochemical characterization of Mustard Oil (*Brassica campestris* L.) with special reference to its fatty acid composition

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ABSTRACT: Edible vegetable oils are the chief source of nutritionally required fatty acids in human diet. Mustard oil, soybean oil, sunflower oil, sesame oil, coconut oil and groundnut oil are among the edible vegetable oils mostly consumed in India. However, none of these oils alone provide many of the lipid soluble nutrients as per the recommendation of health agencies. Mustard oil is considered to be an oil that has low saturated fat as compared to other cooking oils. It is basically consist of oleic acid, erucic acid and linoleic acid. It is also loaded with essential vitamins. Mammalian cells have evolved to de-saturate and elongate α-linolenic acid (ALNA), an omega-3 fatty acid, into eicosapentanoic acid (ω-3) and the longer docosahexanoic acid (ω-3) when the ω-6/ω-3 ratio is low. Excess ω-6 fatty acids in diet saturate the enzymes and prevent the conversion of ALNA into longer forms. The optimal ω– 6/ω-3 ratios that have been estimated to be 2:1 to 3:1 are about four times lower than the current intake. Health agencies, therefore, recommend that human diet should contain more ω-3 fatty acids. Further, studies on the fatty acid composition of oils claiming health benefits have shown that these oils contain lower amount of saturated fatty acids as compared to the unsaturated fatty acids. In the present study, the samples were analyzed for fatty acids, free fatty acids, and peroxide value, iodine number, saponification number were determined according to the standard procedures.

Keywords: Mustard; *Brassica campestris*; omega-3 fatty acid; omega-6 fatty acid; Chromatography: saponification.

INTRODUCTION

Vegetable oils in human diet constitute an important source of energy and have considerable importance in human health. Besides being the gastronomic delights and a source of energy, edible vegetable oils provide fat-soluble micronutrients and essential and nonessential fatty acids, which perform various functions. Besides the fatty acids, unsaponifiable matter is a major chunk of edible vegetable oils. This includes various micronutrients such as tocopherols, tocotrienols, β-carotene, oryzanol, squalene etc. depending on the type of the edible oil. The micronutrients in vegetable oils have various functions. Tocopherols, for example, are recognized for the antioxidant activity, and have been observed to be useful in degenerative diseases like cancer, cataract, cardiovascular disease and ageing. Tocopherols in oil act through several mechanisms such as inhibiting the lipid peroxidation, chain termination, singlet oxygen quenching, and radical scavenging to deactivate free radicals that are produced during the oxidation of biomolecules (Qureshi *et al.*, 1991). Micronutrients also have other roles. The β-carotene, for example, has been recognized for its ability to contribute to the synthesis of retinol (Groff *et al.*, 1995). Oryzanol is known for its strong hypocholesterolemic properties and other beneficial effects (Rukmini & Raghuram, 1991).

Mustard oil is extracted from the seeds of mustard plant (*Brassica campestris* L.), belonging to the cruciferous family of plants. It is originally from the Mediterranean regions, but it grows easily in many parts of the world. It has a characteristically pungent flavor and aroma. Though this oil is nutty tasting it is good for heart and also has many other benefits. Mustard oil contains a high amount of selenium and

magnesium, which gives it anti-inflammatory properties. It also helps stimulating sweat glands and helps lowering body temperature. In traditional medicines, it is used to relieve the pain associated with arthritis, muscle sprains and strains. Seed paste applied on wounds whereas paste of leaf said to heal cattle wounds (Sood *et al.*, 2010). Mustard oil consists of fatty acids like omega alpha 3 and omega alpha 6 which have beneficial properties. Speciality oils having high amounts of a specific fatty acid are of immense importance for both nutritional and industrial purposes. Oil high in oleic acid has demand in commercial food-service applications due to a long shelf-life and cholesterol-reducing properties. Both linoleic and linolenic acids are essential fatty acids; however, less than 3% linolenic acid is preferred for oil stability. High erucic acid content is beneficial for the polymer industry, whereas low erucic acid is recommended for food purposes. Therefore, it is important to undertake systematic characterization of the available gene pool for its variable fatty acid profile to be utilized for specific purposes (Kaushik and Agnihotri, 2000).In the present study the mustard germplasm was analysed by GLC to study its biochemical characterization and fatty acid composition. The GLC analysis revealed that the mustard crop being commonly grown in India are characterized by high Erucic acid content (42.16) in the oil with low levels of Myristic acid (0.58).

MATERIAL AND METHODS

Edible vegetable oil (Mustard oil) used in the present study was extracted from the appropriate source, stored in amber coloured glass bottles, and used within three months of extraction. Mustard oil was extracted from the seeds of *Brassica campestris*, and the oil content was determined by the rapid gravimetric method (Kartha *et al*., 1974).

Oil content is defined as the total oil extractable by n-hexane under specified conditions. Briefly, 0.3 g seeds were weighed and transferred to a glass mortar, and 0.2 g each of glass powder (Pyrex glass washed with concentrated hydrochloric acid) and anhydrous sodium sulphate were added and the mixture was reduced to fine powder. The mixture powder was transferred to a small percolator (20 cm long and 1.5 cm in diameter). The mortar and pestle were washed twice with 0.5 g of anhydrous sodium sulphate and the washings were also packed over the seed powder. Finally, the mortar and pestle were washed with 3 ml of fresh distilled ether (boiling point, 70-90^{0}C) and the washing was added to the packed powder. The mixture was allowed to remain as such for 5 minutes and then percolated by adding 7 ml solvent on top of the column.

The extract was collected in a pre-weighed disk containing one-inch square strip of filter paper. The disk was kept in an oven at 60^{0}C for half an hour to evaporate the solvent. The disk was re-weighed, and difference in the weight of the disk before extraction and after extraction of oil was taken as the weight of the extracted oil. The oil content was expressed in percentage of seed weight. Oil samples were subjected to the following chromatographic and chemical analysis:

1. GLC analysis of fatty acids and Mustard oil sample: The methyl esters of fatty acids in oil samples were prepared by the esterification method proposed by Morrison and Smith (Morrison and Smith, 1964) with slight modification. Briefly, 0.1 ml oil and 0.5 ml of 0.5 N sodium methoxide (prepared in methanol) were mixed in air tight, capped glass tubes. The content of the tubes were mixed thoroughly and heated in boiling water bath for 10 minutes. Tubes were cooled in cold water. Two drops of BF_3 (prepared in 14% methanol) were added to the solution in each tube. Tubes were sealed and heated for 10 minutes in boiling water bath, and then cooled in cold water. After cooling, 5 ml hexane was added to each tube and mixed thoroughly. The mixture was allowed to separate for about 10 minutes, and the upper layer containing the methyl ester was taken out, concentrated and kept in a separate vial for gas chromatography. The esters were analyzed on a Nucon Model gas chromatograph (GC) fitted with flame ionization detector and a column of 15% diethylene glycol succinate (DEGS) coated on chromosorb-W.

Nitrogen was used as a carrier gas at a flow rate of 25 ml/min and the oven temperature was maintained at 180-230°C. Chromatogram of fatty acid standards subjected to GLC analysis is shown in Figure 1.

2. Chemical analysis of free fatty acid in oil samples: Free fatty acid (FFA) content of the oil samples was determined according to the standard protocol of the International Union of Pure and Applied Chemistry (IUPAC, Standard Methods, 1987, 7th edition). Free fatty acid is defined as the number of milligrams of KOH required to neutralize the free fatty acids in 1 g of the oil sample.

The acid value is a measure of the extent to which the triglycerides in the oil have been decomposed. Briefly, 2 g oil sample was weighed and 30 ml of methanol was added to it. The mixture was heated till bubbles appeared. A drop of phenolphthalein indicator was added to the mixture and titrated against 0.1 N KOH. The endpoint of titration was the appearance of a persistent pink colour. FFA was calculated as follows:

$$\text{FFA (as \% oleic acid)} = \frac{M \times N \times 56.1}{W}$$

(*Where, M is the titre value (ml of alkali used up), and N is the Normality of KOH; W: Weight of sample in g*)

Further, free fatty acid in oil is estimated by titrating it against KOH in presence of phenolphthalein indicator, and the acid number is defined as the mg KOH required neutralizing the free fatty acid present in 1g of sample. However, the free fatty acid content is usually expressed as oleic acid equivalents.

3. Iodine number: The standard IUPAC procedure was used to determine the iodine number (IUPAC, Standard Methods, 1987, 7th edition). This number, also known as the iodine value, is an index of the number of double bonds in fat, and therefore, quantifies the degree of unsaturation of fat. Iodine value is reported in terms of the grams of iodine that will react with 100 gram of oil or fat under specified conditions.

Oil is mixed with iodine monochloride (ICl) solution to halogenate double bonds in a fat or oil. The double bonds present in the unsaturated fatty acids react readily with iodine to form an addition compound. Excess iodine monochloride is reduced to free iodine in presence of potassium iodide (KI) and free iodine is measured by titration with sodium thiosulphate using starch as an indicator.

The sample, 0.2 g, is weighed accurately into an iodine flask and dissolved in 20 ml carbon tetrachloride followed by the addition of 25 ml Wijs reagent. The flask is stoppered and the mixture is shaken gently and placed in dark for one hour. At the end of one-hour period, 15 ml of potassium iodide solution is added, and titrated against standardized sodium thiosulphate solution using starch as an indicator until the blue colour disappears. Blank is titrated separately under similar conditions. The iodine number is calculated as follows:

$$\text{Iodine value} = \frac{(B -) \times N \times 12.69}{W}$$

(*Where, B is the volume of 0.1 N $Na_2S_2O_3$ required to titrate the blank (ml), S is the volume of 0.1 N $Na_2S_2O_3$ required to titrate the sample (ml), 12.69 i the atomic weight of iodine and N is the normality of $Na_2S_2O_3$. W is the weight of the oil sample in grams*.)

Oil contains both saturated and unsaturated fatty acids. Iodine gets incorporated into the fatty acid chain wherever the double bonds exits. Hence the measure of iodine absorbed by oil is an indication of the degree of unsaturation, and the iodine value is the gram of iodine absorbed by 100g of the oil. Iodine number indicates the degree of unsaturation of the fat. *Higher the iodine number more is the degree of unsaturation.*

4. Saponification Number: Saponification number/value was calculated according to the IUPAC procedure (IUPAC, Standard Methods, 1987, 7th edition). This value is defined as the number of mg of KOH required to saponify one gram of oil or fat dissolved in solvent. Briefly, 2.5 gram oil is weighed, and mixed with 25 ml (0.5 N) ethanolic KOH, and refluxed for about an hour. After refluxing, the solution is titrated against HCl using phenolpthalein as indicator. Disappearance of pink colour is taken as the

end-point. Blank is titrated separately under similar conditions. Saponification number is calculated using the following formula:

$$\text{Saponification value} = \frac{28.1 \times N \times (X-V)}{W}$$

(*Where, N is the normality of HCl, X is the volume of HCl used in blank (ml), V is the volume of HCl used in sample (ml),28.1 is the* Equivalent weight of potassium hydroxide*, and W is the weight of oil sample in grams*.)

The saponification value gives an indication of the nature of the fatty acids found in the oil sample, and the average chain length of fatty acids present in the fat/oil since the longer the carbon chain the less acid is liberated per gram of fat hydrolysed. *Higher the saponification number, the shorter (or smaller) will be the chain length of fatty acid and vice versa.*

5. Peroxide value: Peroxide value (PV) was determined according to the IUPAC titrimetric method. Briefly, 5 g oil, 30 ml of glacial acetic acid-chloroform mixture (3:2; v/v) and 0.5 ml saturated KI were added and kept in dark for 1 minute. The solution was then titrated against standard $Na_2S_2O_3$ solution using starch indicator.

$$PV = \frac{N(Na_2S_2O_3) \times V(Na_2S_2O_3) \times 1000}{\text{Wt. of the sample}}$$

(*Where, N is the normality of* $(Na_2S_2O_3)$*, V is the volume of* $(Na_2S_2O_3)$ test blank.)

Rancidity can be oxidative rancidity (caused by air) or kenotic rancidity (produced by the microorganisms). In oxidative rancidity, oxygen is taken up by the fat/oil sample and results in the formation of peroxides. The degree of peroxide formation and the time taken for the development or rancidity differs among oils. Peroxide value of oil during oxidative storage is quite common. The peroxide value measures hydroperoxides formed by the reaction of unsaturated oils with triplet or singlet oxygen. The peroxide value is expressed as milliequivalents (mEq) of peroxides per kilogram of oil, and is an index to the oxidative sate of the oil/fat sample.

RESULTS AND DISCUSSION

The samples were analyzed for fatty acids, free fatty acid (as % oleic acid), peroxide value, iodine number and saponification number.The results depicted in Figure 2 show the different fatty acids area of peak in gas chromatogram of mustard oil.

The GLC analysis (Table 1) revealed that the mustard oil is characterized by high Erucic acid content (42.16) with low levels of Myristic acid (0.58).

Oleic acid is present in all foods that contain fat and is easily produced by the fatty acid synthetic pathways present in normal human hepatic cells. It constitutes 15% of the fatty acids in erythrocyte membranes. Oleic acid is the principal lipid of a class that makes LDLs resistant to oxidation and thus a diet rich in this fatty acid reduces foam cell accumulation rates and thereby lowers the chances of atherosclerosis. Many plants produce this polyunsaturated fatty acid, but because of the small amounts of fresh vegetables consumed, it is one of the least abundant of the essential fatty acids in most diets. It is found in flax, hemp, rape (canola) seed, soybean, walnut oils, and dark green leaves and must be supplied by such foods (Lopez *et al*., 2005).

Linoleic acid (LA) is by far the most abundant polyunsaturated fatty acid in most human tissues (Whelan, 2008). It is one of the essential fatty acids, because it contains a double bond at the omega-6 position, which is beyond the reach of the human desaturase enzyme. Low levels indicate dietary insufficiency that leads to a variety of symptoms. Some of these symptoms result from lack of linoleic acid in membranes where it serves a role in structural integrity. Most, however, are from failure to produce the 1-series and 3-series local hormones known as prostanoids (Khulusi *et al*., 1995). Showing association of clinical symptoms with dietary insufficiencies indicates the central importance of α-linolenicacid (ALA) and its

counterpart γ-linolenicacid (GLA). The wide range of symptoms is due to the function of ALA in critical cell processes of membrane integrity and eicosanoid local hormone production. The latter function utilizes the fatty acid, eicosapentaenoic acid (EPA) that can be produced from ALA by elongation and desaturation (Wathes *et al*., 2007).

Insufficiencies of eicosapentaenoic acid are likely the most prevalent fatty acid abnormality affecting the health of persons in Western societies. Low levels in plasma, or especially in erythrocytes, are indicative of insufficiency. Arthritis and heart disease, as well as aging, result from direct or indirect effects of unchecked inflammatory response. Supplementation with EPA-rich fish oils aids in the prevention of cardiac arrhythmias (Nair *et al*., 1997). Significant reduction in total cholesterol and triglyceride has been achieved with a combination of garlic concentrate (900 mg/d) and fish oil supplementation (Adler & Holub, 1997).

The growth and development of the central nervous system is particularly dependent upon the presence of an adequate amount of the very long chain, highly unsaturated fatty acids, docosapentaenoic (DPA) and cervonic acid (DHA) (Innis, 1992; Hoffman *et al*., 1993). Attention deficit hyperactivity disorder (Stevens *et al*., 1995) and failures in development of the visual system in essential fatty acid deficiencies are two examples of this dependency. Cervonic acid (DHA) is an important member of the very long chain fatty acids (C22 to C26) that characteristically occur in glycosphingolipids, particularly those in brain tissue. Since this fatty acid is so important in early development, it is worth noting that the levels in breast milk are correlated with the mother's intake of fish oils (Henderson *et al*., 1992) which are rich sources of cervonic acid and docosapentaenoic (DPA).

Stearic acid is a saturated fatty acid that is two carbon atoms longer than palmitic and is similarly cholesterogenic. High levels in plasma are therefore a risk factor in atherosclerotic vascular disease. Abnormal levels in erythrocyte membranes cause alteration in membrane fluidity with numerous consequences Low levels give increased fluidity, which is associated with active tumor proliferation (Zamaria, 2004).

The above discussion makes it clear that a balanced composition of the fatty acids in edible oils is essential for health benefits. Table-2 shows the SFA, MUFA, PUFA ratio and the ω-6/ω-3 amount in mustard oil.

Table 1: **Fatty acid composition of mustard oil measured on GLC.**

Fatty acid	MO
Myristic acid (C14:0)	00.58
Palmitic acid (C16:0)	01.74
Palmitoleic acid (C16: 1)	00.17
Stearic acid (18:0)	01.04
Oleic acid (18:1) [1]	09.56
Linoleic acid (18:2) [2]	13.31
Linolenic acid (18:3) [3]	11.10
Eicosanoic acid (C20:0)	06.36
Eicosadienoic acid	--
Behenic acid (C20: 1)	01.65
Erucic acid (C22:0)	42.16
Lignoceric acid (C22: 1)	2.23

Table 2: SFA, MUFA, PUFA ratio and the ω-6/ω-3 ratio in mustard oil

Fatty Acid	SFA	MUFA	PUFA	SFA:MUFA:PUFA	Ω-6/ω-3 ratio
Amount/Ratio	6.14	69.8	26.7	1 : 11.37 : 4.35	1.14

In human diet, the ω-6/ω-3 ratio is important, as humans evolved by consuming a diet lower in saturated fat and higher in omega-3 fatty acids than is consumed today. Early in the evolution, man lived by the oceans and subsisted primarily on fish or lived inland and consumed large quantities of green plants high in α-linolenic acid. Paleolithic diet, therefore, is thought to have been lower in ω-6 fatty acid sources, and might have ω-6/ω-3 ratio that approximated 1:1. However, the modern diet is richer in ω-6 fatty acids from animal proteins and especially from the oils extracted from grains such as corn and sunflower, with an estimated ω-6/ω-3 ratio of 8:1 to 12:1. Mammalian cells cannot convert ω-3 fatty acid to ω-6 fatty acid but they have evolved to de-saturate and elongate ALNA (ω-3) into eicosapentanoic acid (ω-3) and the longer docosahexanoic acid (ω-3). However, this conversion is possible only when the ω-6/ω-3 ratio is low. Excess ω-6 fatty acids in diet would saturate the enzymes and prevent conversion of ALNA into longer forms (Kris-Etherton & Yu, 1997). The optimal ω– 6/ω-3 ratios that have been estimated to be 2:1 to 3:1 is four times lower than the current intake. Health agencies, therefore, recommend that human should consume more ω-3 fatty acids. In the present study, the ratio of ω-6/ω-3 fatty acids in analysed samples is fairly better.

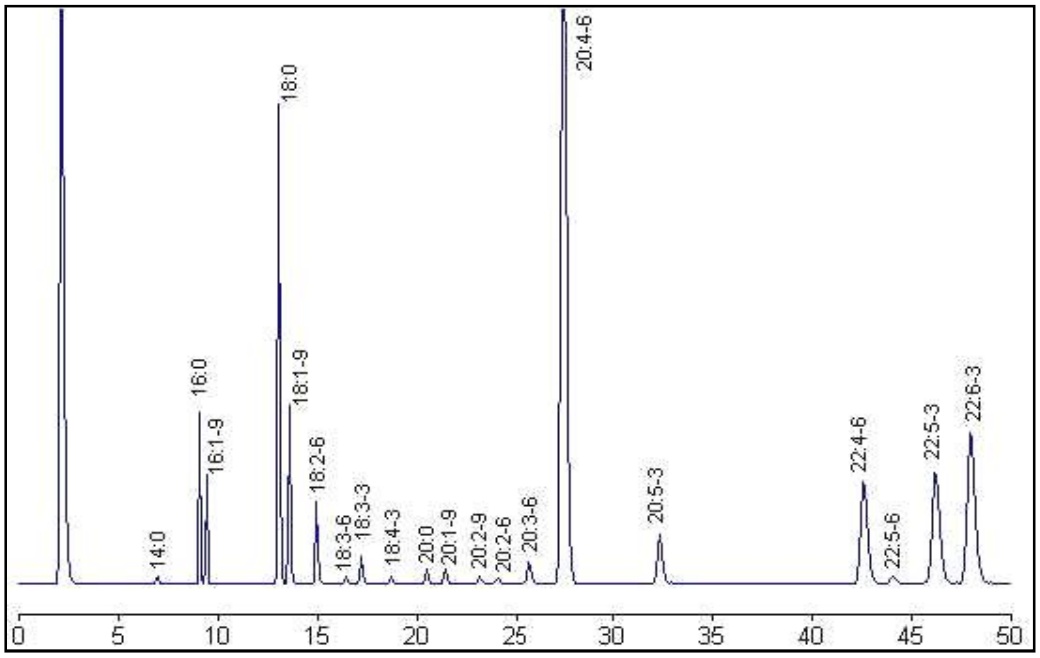

Figure 1: Chromatogram of fatty acid esters prepared from the fatty acid standards procured from Sigma Chem. Co. Peak identification: (14:0) Myristic, (16:0) Palmitic, (16:1-9) Palmitolic, (18:0) Steric, (18:1-9) Oleic, (18:2-6) Linoleic, (18:3-6) Gamma linoleic, (18:3-3) linoleinic, (18:4-3)Stearidonic, (20:0) Archidic, (20:1-9) 11-Ecosenoic, (20:2-9)octadecatrienoic,(20:2-6) Ecosadienoic, (20:3-6) Dhimogamma linoleic, (20:4-6) Archidonic, (20:5-3) Ecosapentaenoic, (22:4-6) Docosatetraenoic, (22:5-6) Docosapentaenoic (Osbond acid),(22:5-3) Docosapentaenoic (Clupanodonic acid), (22:6-3) Docosahexaenoic.

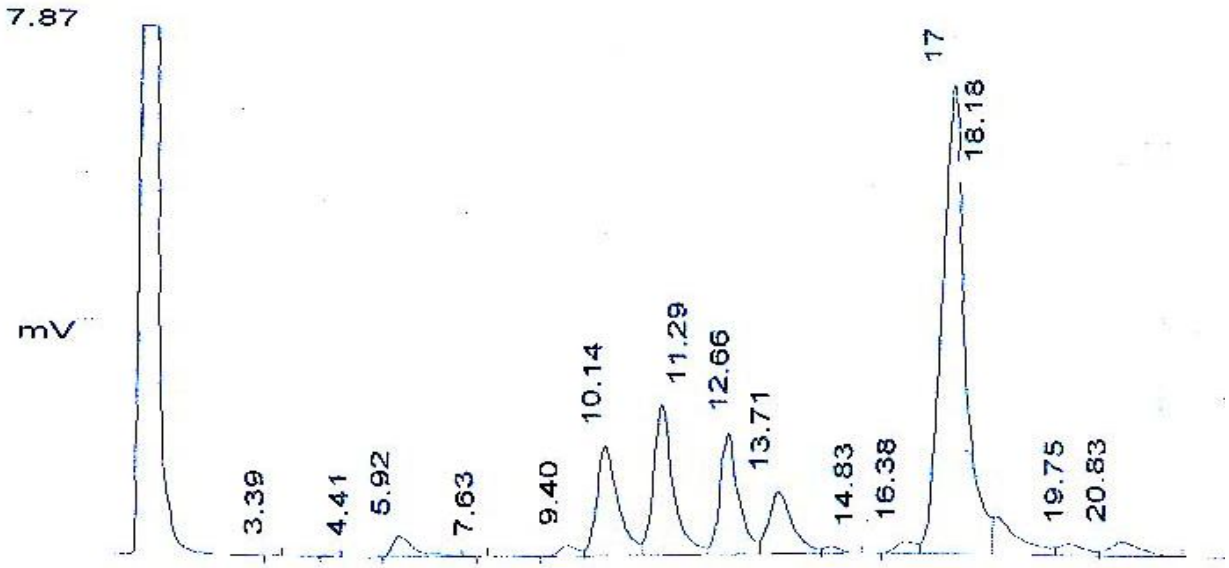

Figure 2: Gas chromatogram of mustard oil. Description of peaks is as follows: (3.39 Myristic acid), (5.92 Palmitic acid), (7.63Palmitolic acid), (9.40 Stearic acid), (10.14 Oleic acid), (11.29 Linoleic acid), (12.66 Linoleinic acid),(13.71Ecosenoic acid), (14.83 Behenic acid), (17.00 Erucic acid).

Chemical analysis of the mustard oil showed that the saponification number, iodine number, peroxide value, acid value, free fatty acid, fatty acids micronutrient analysis were within the limits set for an edible vegetable oil (Figures 3-4).

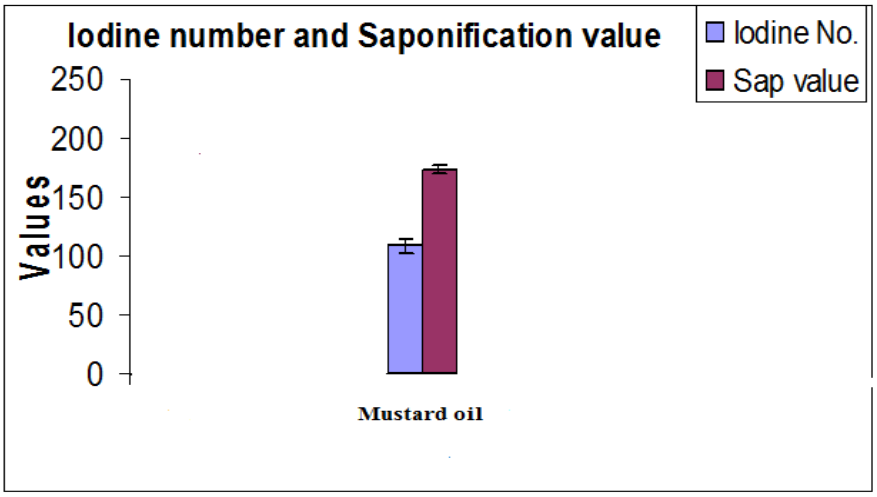

Figure 3: Iodine number, expressed as g iodine/100g of oil, and saponification value, expressed as mg KOH/g oil. Unsaponifiable matter is represented as percent by weight. Mean±S.D. (n=3). Values in brackets show the normal range.

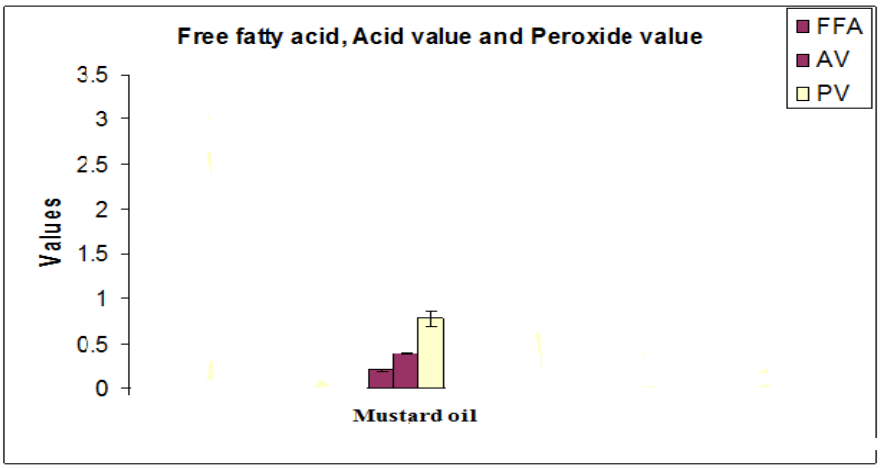

Figure 4: Free fatty acid, expressed as mg KOH/g oil, acid value (AV), expressed as mg KOH/g oil, and peroxide value, expressed as mEq per kg.

CONCLUSIONS

Fats constitute an important constituent of the diet because of their high energy value and because of the fat soluble micronutrients and essential fatty acids. Therefore, both the quality and the quantity of fats in human diet affect health and an imbalance intake may often lead to diseases including chronic debilitating diseases such as the cardiovascular disease and rheumatoid arthritis. Among the fatty acids present in fats and oils are MUFA such as oleic acid and EFA, essential fatty acids are of particular interest. EFAs are polyunsaturated, and include linoleic acid (n-6 or ω-6 fatty acids), and α-linolenic acid (n-3 or ω-3 fatty acids). Essential fatty acids are critical for human survival. In the body, EFAs are metabolized to their respective long-chain metabolites, which have beneficial effects. EFAs form the precursors to respective prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs), and also give rise to lipoxins (LXs), resolvins, isoprostanes, and hydroxy- and hydroperoxyeicosatetraenoates. Certain PGs, TXs, and LTs have pro-inflammatory actions whereas LXs and resolvins are anti-inflammatory in nature. Furthermore, EFAs and their long-chain metabolites modulate the activities of enzymes (angiotensin converting enzyme), enhance acetylcholine levels in the brain, increase the synthesis of endothelial nitric oxide, augment diuresis, enhance insulin action, and may function as endogenous HMG-CoA reductase inhibitors, nitric oxide enhancers, β-blockers, anti-hypertensive, and anti-atherosclerotic molecules. EFAs/metabolites have been reported to react with nitric oxide (NO) to yield respective nitroalkene derivatives that exert cell-signaling actions via ligation and activation of peroxisome proliferator activated receptors. In clinical practice, conditions have been reported in which EFAs, especially the n-3 series have been shown to have synergistic effects with drugs; examples include: hypertension, arthritis, psoriasis, and ulcerative colitis. Essential fatty acids should be in an optimal ratio in diet. A desirable n-6/n-3 ratio is in the range of 5 – 10. A ratio above 50 is injurious to health. Further, health agencies such as WHO and American Heart Association recommends that fats and oils should not supply more than 30% energy of diet and that the fatty acid composition in oil and fats should have a SFA, MUFA, PUFA ratio of 1: 1.5: 1. A higher MUFA in oils and fats is recommended for health benefits.

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