BIOCHEMICAL EVALUATION OF SOME OILS RICH IN ω-3 OR γ-LINOLENIC ACIDS AND THEIR ANTICANCER ACTIVITY

Journal

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ABSTRACT

The influence of diets supplemented with evening primrose seeds oil (EPO) or marine alga oil (MO) or linseeds oil (LO) on serum lipid profile, liver and kidney functions in rats were studied. Furthermore the anticancer activity; cytotoxicity of the three on breast, larynx, colon and liver human tumor cell lines tested using sulphorhodamine B (SRB) assay were also studied representing different cancer types. Results revealed that, the serum total cholesterol concentrations of rats fed the EPO or MO or LO diets were significantly lower and the MO diet was the best one in all groups after 6 weeks. Also, HDL-cholesterol levels were significantly increased in all groups; except the 3 week MO group, as compared to the control group. Moreover, it was observed that EPO after 6 week had the highest value.

Triglycerides concentrations in the EPO and LO groups significantly decreased, whereas no significant trend was observed in the MO group. In addition, the supplementations of EPO, MO and LO have no significant effect on liver and kidney functions.

The linseed oil exhibited an efficient cytotoxicity against breast (MCF7), larynx (HEP2) and liver (HEPG2) carcinoma cell line with IC50 values of 4.4, 5.23 and 2.27µg, respectively

and did not have cytotoxic effect against colon carcinoma cell line, while MO and EPO did not maintain any cytotoxic property against all tumor cell lines under our experimental conditions.

INTRODUCTION

Evening primrose seed oil (EPO) is a highly unsaturated oil (91.25%), distinguish with a high content of γ-linolenic acid (all-cis-6,9,12-octadecatrienoic acid –GLA), approximately 11±3% of the total fatty acids in the oil is GLA(C18:3n-6). GLA has physiological functions of modulating immune and inflammatory responses and is effective for treating certain skin-related disorders (i.e., atopic eczema, rheumatoid arthritis, Eid et al. (2007) as well as diabetic neuropathy and premenstrual syndrome. Also studies have shown that GLA can help people with breast cancer, cardiovascular diseases, high blood pressure, Erdemoglu et al. (2004) and Kotnik et al. (2006).

Dietary marine oils are known with their high content of long-chain n-3 polyunsaturated fatty acids, mainly eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Omega-3 EPA and DHA intake may prevent cardiovascular diseases, certain types of cancer, inflammations and allergies as well as improve proper development and function of central nervous system Kolanowski (2006). The ω3 family, DHA; 22:6n3 is of particular interest because it has been shown to be essential for optimal brain and eye functions. DHA is an important building block of the brain, nerves and eyes. The brain itself is made up of 60% lipid, and DHA is the most abundant fatty acid in both the brain and the retina Samah(2003). Besides changing of eating and culinary habits, the increase of omega-3 PUFA level in a diet may be achieved by intake of fish oil supplements or food enriched with fish oil Metcalf et al.(2003). Therefore, a number of functional foods enriched with omega-3 PUFA via fish oil addition have been developed.

Humans have been eating flax for thousands of years. Flax was valued in Ancient and Early Modern times as a food and medicine Morris(2009a). Today, consumers turn to flax for its pleasant, nutty flavor and many health benefits due to its high alpha linolenic acid ALA content, flax oil has an omega-6/omega-3 fatty acids ratio of 0.3:1, and its anti-inflammatory effect. Eating flax regularly, improves laxation, lowers blood cholesterol, aids in blood glucose control, and blocks inflammation Zhao et al.(2004), and may help prevent or treat chronic diseases in which inflammation plays a role in chronic

diseases like heart disease, stroke, diabetes, cancer, obesity, the metabolic syndrome, and Alzheimer disease Morris(2009b).

Carcinogenesis is a multistage process which frequently depends on the environmental agents. It is considered that 35–40% of all human cancers may be associated with the diet Peto (2001) and dietary fat is regarded as one of the major risk factors in cancers of the breast, colon and prostate Woutersen et al. (1999). It has been reported that fats containing high concentrations of n-6 polyunsaturated fatty acids (n-6 PUFA), especially linoleic acid (LA; 18:2), promoted the development of mammary tumours Zock and Katan (1998). On the other hand, evening primrose oil (EPO) containing about 75% of linoleic acid and a relatively high level (9%) of γ-linolenic acid (GLA; 18:3, n-6) was reported to inhibit the development of carcinogen-induced rat mammary tumours Abou-El-Ela et al. (1988).

In contrast to fat containing n-6 PUFAs, fish oils (FO) rich in n_3 PUFAs, the most abundant being EPA; 20:5 and DHA; 22:6, were observed to inhibit carcinogen-induced mammary tumorigenesis in the rat and mouse Caygill et al. (1996).

The present investigation was carried out to study the effect of EPO, MO and LO on the serum lipid profile, liver and kidney functions of rats. Besides, efficiency of these oils as anticancer agents for breast, larynx, colon and liver were evaluated.

MATERIALS AND METHODS

Oils

Evening primrose *Oenothera biennis* L. seeds were obtained from Medicinal and Aromatic Plants Dept., Horticulture Res. Inst., Agricultural Res. Center. Linseeds *Linum usitissimum* L. were obtained from Fiber Res. Section, Field Crops Res. Inst., Agricultural Res. Center, Egypt. Marine oil (MO) (Martek DHA-S), nutritional oil derived from the marine alga, a rich source of omega-3 docosahexaenoic acid (DHA) was obtained from Martek Bioscience Corporation, USA.

Chemicals

All chemicals and Solvents used were of analytical grade and obtained from Sigma Chemical Co. (London, Ltd. Poole), England. Reagents Methodology Kits were obtained from Biodiagnstic Research Reagents Co., Egypt,

Animals

Male white albino rats of Sprague-Dawely strains of 120 -135 g body weights were used in this study. The animals were kept individually in stainless steel cages at air condition 20-22º C and a relative humidity of about 55%.

Diet

A basal diet composed of 15% casein, 10% corn oil, 65 % starch, 5% fiber, 4% salt mixture and 1% vitamin mixture (Compbell, 1961) were prepared for feeding all groups of rats throughout the experiment period.

Experimental Design

Twenty eight male Sprague-Dawley rats were used in this study. The rats were divided into four groups (n=6), which were fed *ad libitum* the rat basal diet. After one week adaptation to the experimental regimen, the control group fed the basal diet throughout the experiment period, while, the other three groups fed the same basal diet except for using 9% corn oil only and administrated 1 ml of the tested oils daily (each in its group) by intragastric intubations. The diet was freshly prepared every five day, the diet and tested oils stored at –20 °C during the feeding period. The rats received fresh food daily. After 45 days of experiment rats were killed by decapitation and liver tissue was excised, rinsed thoroughly with ice-cold physiological saline and kept frozen at -20°C until further analysis.

Oil extraction

Dried evening primrose seeds were ground in laboratory mill and soaked in n-hexane for 24 hrs twice. Solvent was collected and evaporated under vacuum. While, the crushed linseeds sample was pressed with laboratory type of Carver hydraulic press under 10.000 lb/in pressure for 1 hr at room temperature. The produced oils were filtered and kept in dark bottles in the refrigerator until used.

Gas chomatography analysis for fatty acids

Methylation of fatty acids

An aliquot of oils, about 10mg, was dissolved in 2ml hexane and then 0.4ml 2N KOH in anhydrous methanol was added Cossignani et al. (2005), after 3 min, 3ml water was added. The organic layer, separated, dried over anhydrous sodium sulfate, then concentrated

with a N_2 stream to around 0.5 ml for GC analysis of fatty acids methyl esters (FAME) as described below.

Identification of fatty acids methyl esters by GLC

Agilent 6890 series GC apparatus provided with a DB-23 column (60m x 0.32mm x 0.25μm). Fatty acids methyl esters directly injected into the GC. Carrier gas was N_2 with a flow rate of 2ml/min, splitting ratio of 1:100. The injector temperature was 250^{o}C and that of FID detector was 270^{o}C. The temperature settings were as follows: 150^o to 225°C at 5^{o}C/min, and then held at 225^{o}C for 20 min . Peak identification was performed by comparison of the retention time (RT) for each peak with those of standard fatty acids. The peaks areas were measured using Chemstation Program, and relative areas of the identified fatty acids were recorded.

Blood sampling

Blood samples were taken at 3 and 6 weeks. The blood samples were obtained from orbital plexus venous by means of the fine capillary glass tubes according to the method described by Schermer (1967). Each sample was placed in a dry and clean centrifuge tube and allowed to clot (undisturbed) for 1-2 hr. At 37^{o}C, serum was then removed using a Pasteur pipette and centrifuged for 10 min at 3000 rpm to remove any suspended red blood cells. The clean homogenized supernatant serum was then pipetted into epindorff tubes. Serum from 2 rats was pooled together so that 3 sets of data were produced for each groups and kept frozen until analysis.

Lipid extraction

Liver total lipid was extracted with a mixture of choloroform/methanol (2:1v/v) as described by Kates (1986). The separated lipid was transformed into methyl ester and analysed by GC.

Serum analysis

The methods reported by Fawcett and Soctt (1960) and Schirmeister (1964) were used for determination of Urea and Creatinine. Aspartate (AST) and alanine (ALT) amino transferase activites were determined following method of Reitman and Frankel (1957). The activity of alkaline phosphatase was determined according to the method of Belfield and Goldberg (1971). Enzymatic colorimetric methods were used for estimation of Triglycerides,

Cholesterol and HDL- cholesterol by Fassati and Prencip (1982) , Richmond (1973) and Burstein (1970), Respectively.

Statistical analysis

The collected data of biological examination were statistically analyzed. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's Multiple Range test.

Measurement of potential cytotoxicity by sulphorhodamine B (SRB) assay

Potential cytotoxicity of MO, EPO and linseed oils were tested using the method of Skehan et al. (1990) in National Cancer Institute, Egypt.

Cells for breast (MCF7), colon (HCT), larynx (HEP2) or liver (HEPG2) cancer were plated in 96-multiwell plate (10^4cells/well) for 24 hours before treatment with the tested oils to allow attachment of cell to the wall of the plate. Different concentrations of the oils under test (0, 1, 2.5, 5 and 10μg/ml) were added to the cell monolayer.

RESULTS AND DISCUSSION

Results

Fatty acids composition of experimental oils

The main differences among the diets were the contents of γ-linolenic acid in EPO (8.64%), eicosapentaenoic acid EPA (5.78%) and docosahexaenoic acid DHA (13.86%) in MO. Meanwhile, the high contents of alfa linolenic acid in LO (55.04%) was noticed in Table (1).There were differences in the levels of SFA and USFA in diets, it were 8.73, 21.87, 10.93 and 82.64, 63.72, 89.08% for EPO, MO and LO, respectively.

Fatty acid composition of liver

Table 2 shows the effect of dietary lipids, evening primrose oil, marine oil and linseed oil on fatty acid composition of rat liver total lipids. The type of dietary lipids affected the fatty acid composition. Livers of EPO, MO and LO rats exhibited liver levels of 18:2 fatty acid compared to control rats. EPO group increase the level of γ-linolic acid in the rat liver compared to control group. While, LO group increase the level of ∝-linolenic acid compared to control

group. On the other hand, MO diet rats had higher proportions of 20:2, 20:4, 22:4, 22:5 and 22:6 compared to control group.

Table (1) Fatty acid composition of the experimental oils

Fatty acid	Corn oil	EPO	MO	LO
14 : 0	nd	Nd	3.45	Nd
16 : 0	11.59	7.51	16.37	6.04
16 : 1	0.15	Nd	0.28	0.09
18 : 0	1.89	1.22	1.52	4.89
18 : 1	29.59	13.41	19.99	19.33
18 : 2	56.06	69.05	36.05	14.62
18 : 3 (γ n-6)	nd	8.64	Nd	nd
18 : 3 (αn-3)	0.71	0.18	0.53	55.04
20 : 0	nd	Nd	0.44	nd
20 : 2	nd	Nd	0.61	nd
20 : 3	nd	Nd	0.22	nd
20 : 4	nd	Nd	0.26	nd
20 : 5	nd	Nd	0.56	nd
22 : 5	nd	Nd	5.78	nd
22 : 6	nd	Nd	13.86	nd
SFA	13.48	8.73	21.87	10.93
USFA	86.51	82.64	63.72	89.08

nd: Not detected SFA: Saturated fatty acid USFA: Unsaturated fatty acids

Table (2) Fatty acid composition of liver total lipids in rats fed evening primrose (EPO) oil, Marine alga oil (MO) and linseed oil (LO) for 45 days

Fatty acid	Control	EPO	MO	LO
14 : 0	1.24	0.48	1.11	0.85
16 : 0	20.51	20.94	25.93	23.68
16 : 1	1.89	1.35	1.82	1.91
18 : 0	17.61	15.61	14.84	15.14
18 : 1	14.65	12.78	15.92	19.30
18 : 2	15.62	25.13	25.55	20.15
18 : 3 (γ n-6)	nd	4.73	Nd	nd
18 : 3 (αn-3)	0.89	0.32	0.79	5.91
20 : 0	16.7	16.17	0.25	6.68
20 : 1	nd	2.54	0.40	nd
20 : 2	nd	Nd	1.00	nd
20 : 5	nd	Nd	0.51	nd
22 : 4	nd	Nd	0.79	nd
22 : 5	nd	Nd	0.95	nd
22 : 6	nd	Nd	4.26	nd
SFA	55.83	8.73	42.13	46.35
USFA	33.05	82.64	53.07	47.27

nd: Not detected SFA: Saturated fatty acids USFA: Unsaturated fatty acids

Total Cholesterol

A Significant overall decrease in total cholesterol level was observed, except the 3 week LO group (Table 3). The serum cholesterol levels of rats fed the EPO, MO and LO diets also were significantly lower than that in rats feed control diet, it was showed that MO diet was the best one in all groups after 6 weeks.

Triglycerides

The changes in triacylglycerol concentrations are significantly differed between the groups (Table 3). In the EPO and LO groups

concentrations significantly decreased, whereas no significant trend was observed in the MO group.

Table (3) Effect of dietary EPO, MO and LO oils on cholesterol, triglycerides and HDL-cholesterol concentrations of rats

Groups	Period (Week)	Cholesterol mg/dL	Triglycerides mg/dL	HDL-cholesterol mg/dL
Control	3	91.26 ± 3.61^{a}	175.93 ± 7.94^{a}	81.76 ± 4.45^{b}
	6	91.29 ± 3.61^{a}	175.95 ± 7.94^{a}	81.74 ± 4.45^{b}
EPO	3	36.40 ± 1.30^{d}	131.66 ± 13.25^{b}	92.06 ± 2.90^{a}
	6	43.00 ± 5.20^{c}	107.00 ± 5.00^{c}	132.80 ± 5.23^{c}
MO	3	57.30 ± 2.80^{b}	170.90 ± 4.64^{a}	81.53 ± 5.61^{b}
	6	23.10 ± 1.75^{e}	175.00 ± 5.00^{a}	96.40 ± 5.00^{a}
LO	3	95.16 ± 0.05^{a}	171.00 ± 5.00^{a}	98.20 ± 5.50^{a}
	6	34.46 ± 4.60^{d}	108.60 ± 5.30^{c}	123.50 ± 8.15^{c}

Values are expressed as means±SEM
Values on the same column not sharing the same superscript letters were significantly different ($P<0.05$), n=6 rat

HDL-cholesterol

HDL-cholesterol levels were significantly increased in all groups; except the 3 week MO group, as compared to the control group. It was observed that EPO after 6 week had the highest value (Table 3).

As shown in Table 4, and 5, it was concluded that the supplementations of EPO, MO and LO have no significant effect on liver and kidney function.

Table (4) Effect of dietary EPO, MO and LO oils on AST, ALT and ALP concentrations of rats

Group	Period (Week)	AST U/ml	ALT U/ml	ALP U/L
Control	3	52.00 ± 5.29^{ab}	37.33 ± 1.15^{a}	297.86 ± 31.99^{bc}
	6	52.10 ± 5.29^{ab}	37.30 ± 1.15^{a}	297.89 ± 31.99^{bc}
EPO	3	41.33 ± 6.11^{bc}	34.00 ± 3.46^{b}	333.26 ± 21.77^{abc}
	6	43.00 ± 1.00^{bc}	34.33 ± 0.57^{b}	345.26 ± 48.96^{ab}
MO	3	52.00 ± 5.29^{ab}	37.21 ± 1.00^{a}	373.26 ± 25.10^{a}
	6	42.21 ± 2.00^{bc}	37.50 ± 1.15^{a}	287.2 ± 10.4^{c}
LO	3	58.00 ± 11.13^{a}	37.33 ± 0.57^{a}	324.6 ± 20.2^{abc}
	6	41.11 ± 1.00^{bc}	34.22 ± 0.64^{b}	344 ± 15.24^{ab}

Values are expressed as means±SEM
Values on the same column not sharing the same superscript letters were significantly different (P< 0.05), n=6 rat

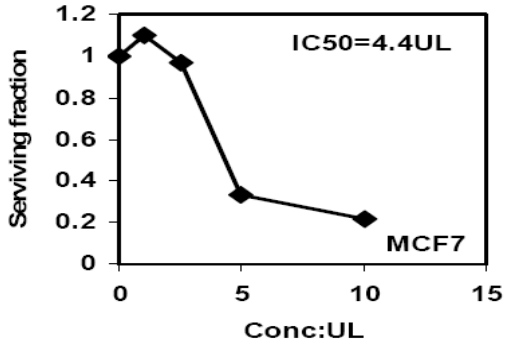
Table (5) Effect of dietary EPO, MO and LO oils on urea and creatinine concentration of rats

Group	Period (Week)	Urea mg/dL	Creatinine mg/dL
Control	3	39.96 ± 5.00^{a}	0.59 ± 0.04^{bc}
	6	39.94 ± 5.00^{a}	0.60 ± 0.04^{bc}
EPO	3	31.66 ± 2.75^{b}	0.64 ± 0.02^{bc}
	6	33.98 ± 1.00^{b}	0.30 ± 0.07^{d}
MO	3	27.16 ± 1.75^{c}	0.67 ± 0.08^{b}
	6	24.56 ± 0.15^{cd}	0.31 ± 0.02^{d}
LO	3	39.30 ± 0.80^{a}	0.56 ± 0.05^{c}
	6	22.73 ± 1.51^{d}	0.54 ± 0.01^{c}

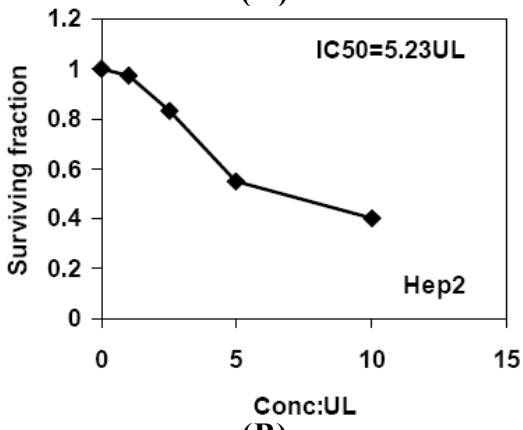
Values are expressed as means±SEM.
Values on the same column not sharing the same superscript letters were significantly different (P<0.05), n=6 rat

Cytotoxicity

MO, EPO and LO were evaluated in National Cancer Institute (Egypt) for its cytotoxicity activity in in-vitro disease oriented antitumor screening using sulphorhodamine B (SRB) assay including 4 human tumor cell lines representing different cancer types.. The results in Fig (1) revealed that linseed oil exhibited an efficient cytotoxicity against 3 human tumor cell lines representing breast carcinoma (MCF7) (Fig 1-A), larynx carcinoma (HEP2) (Fig1.B) and liver tumor cell lines (HEPG2) (Fig1-C) with IC_{50} values of 4.4, 5.23, 2.27μg, respectively, and have no cytotoxic effect against colon tumor cell lines (HCT) Jelinska et al. (2003). On the other hand MO and EPO were proven to have no cytotoxic effect against the 4 tested human tumor cells.



(A)

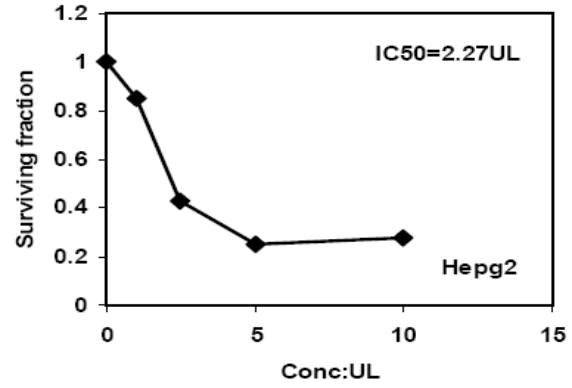


(B)

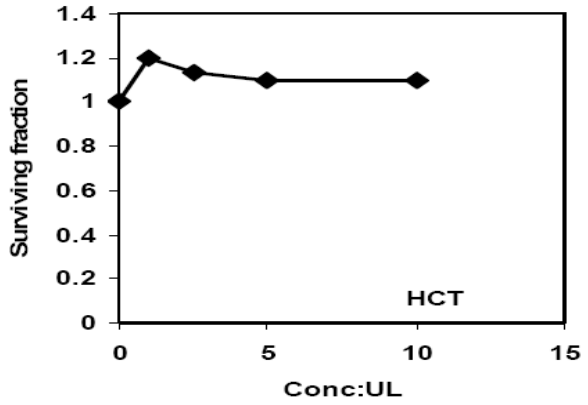
Fig 1 : Cytotoxic activity of LO

(A) against breast carcinomal cell line (MCF7)

(B) against larynx carcinomal cell line (HEP2)



(C)



(D)

Fig 1 : Cytotoxic activity of LO
(C) against liver carcinomal cell line (HEPG2)
(D) against colon carcinomal cell line (HCT)

Discussion

Evening primrose oil contains high level of gamma linolic acid (GLA), which is essential for the synthesis of other PUSFAs. These fatty acids are important building blocks for phospholipids as well as precursors for prostaglandin synthesis. It is generally accepted that PUSFAs intake lower plasma cholesterol levels, diets supplemented with EPO (GLA content 8.64%) reduced the level of cholesterol and our results are confirmed with those obtained by Chan et al. (1991).

DHA is an omega-3 fatty acid. All the fatty acids which are essential in human diet are belong to either omega-3 family or omega-6 family.

MO has lowering effect on total cholesterol and triglycerides and increase the level of HDL-cholesterol. These results are in accordance with others like; Murata et al. (2002) who suggested that the simultaneous consumption of fish oil (rich in EPA and DHA) decreases the concentration of triacylglycerol in the serum and liver.

Our results which showed that linseed oil reduce the cholesterol level and triacylglycerol concentrations are in agreement with those reported by Morris, 2009a, b, c).

Concerning cytotoxicity evaluation of the experimented oils, linseed oil exhibited an efficient cytotoxicity against 3 human tumor cell lines, while MO and EPO had no cytotoxic effect for the 4 tested cell lines with the dosages used. That may attributed to the high unsaturated fatty acids content, consequently a high susceptible to free-radical peroxidation. This means that a higher cell-damaging free radicals are formed, although the almost similarity of fatty acids profile of unsaturated fatty acids and γ-linoleic acid. One possible reason for these results may be, that there is something about the linseed oil specifically and not the unsaturated content, that is influencing breast, larynx and liver cancer risk. Linseed oil contains lignans. Lignans are both antioxidants and phytoestrogens. Antioxidants are compounds that work to keep oxygen from reacting with and damaging proteins, fats and other compounds in our tissues, vitamins, flavonoids, and a phenolic compound that may help to slow down the development of the three above tumor cells.

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التقييم البيوكيماوى لبعض الزيوت الغنية فى محتواها من الأحماض الدهنية اوميجا-3 او جاما لينولينك والنشاط المضاد للسرطان لها

[1]منير محمد عيد، [2]صفاء عزت على و [3]محجوب محمد احمد

[1] قسم بحوث الزيوت والدهون- معهد بحوث تكنولوجيا الأغذية-مركز البحوث الزراعية-جيزة-مصر

[2] وحدة التدريب والتطوير-معهد بحوث تكنولوجيا الأغذية-مركز البحوث الزراعية-جيزة-مصر

[3] الهيئة العامة للرقابة والبحوث الدوائية

اجرى هذا البحث لدراسة تأثير الوجبات المدعمة بزيت بذور الأونثرا او الزيوت البحرية او زيت بذور الكتان على ليبيدات الدم وكذلك تأثيرها على وظائف الكبد والكلى لفئران التجارب . هذا بجانب دراسة تأثير هذه الزيوت كمواد مقاومة لسرطان الثدى و المرئ و القولون و الكبد وذلك بإستخدام خلايا بشرية بواسطة طريقة سلفورودامين- ب .

وقد اظهرت النتائج إنخفاض معنوى فى تركيزات الكوليسترول الكلى فى سيرم الدم لكل الوجبات المستخدمة وكان افضل هذه الوجبات تأثيرا الوجبة المدعمة بالزيوت البحرية طوال فترة التجربة. بينما حدث ارتفاع معنوى فى مستوى الكوليسترول عالى الكثافة لكل المجاميع المختبرة بالمقارنة بالكنترول عدا مجموعة الزيوت البحرية بعد فترة ثلاث اسابيع من التجربة ، وكان اعلى ارتفاع فى مستوى الكوليسترول عالى الكثافة لمجموعة زيت بذور الأونثرا فى نهاية فترة التجربة .

كما حدث إنخفاض معنوى فى تركيزات التراى جليسريد لكل من الوجبات المدعمة بزيت بذور الأونثرا والكتان مقارنة بالوجبة المدعمة بالزيوت البحرية و التى لم يكن لها ثاثير ثابت خلال فترة التجربة .وايضا بينت الدراسة انه لا يوجد اى تاثير على وظائف الكبد والكلى فى فئران التجارب المختبرة.

كما أثبتت النتائج أن لزيت بذور الكتان تأثير واضح في وقف نشاط الخلايا السرطانية لكل من الثدي، المرئ والكبد بقيم IC50 بلغت 4.4، 5.23، 2.27 ميكروجرام، علي التوالي بينما لم يكن له تأثير في وقف نشاط الخلايا السرطانية للقولون. كما أثبتت النتائج ايضا ان زيت الأونثرا والزيوت البحرية لم يكن لها تأثير في وقف نشاط كل الخلايا السرطانية المختبرة بالتركيزات المستخدمة.