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CONJUGATED LINOLEIC ACID: PREPARATION AND BIOLOGICAL EVALUATION

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ABSTRACT

Conjugated linoleic acid (CLA) was successfully prepared using three different substrates; I] dehydration of castor bean oil; 2] isomerization of the separated fatty acids (SFA) after saponification of oil rich in linoleic acid (LA) content and 3] isomerization of commercial linoleic acid.

Dehydration process was carried out at 250±5°C for several hours using phosphoric acid (0.1% w/w) as a catalyst, at which, satisfactory proportions of CLA were obtained. By monitoring the changes in fatty acids composition and other analytical parameters, the highest yield (50.93%) of CLA was attained after only 3 hr of dehydration process.

The SFA rich in LA were subjected to isomerization reaction which was achieved at 120±5°C for 48 hr using alkaline catalyst;(sodium hydroxide in n-butanol) under gentle stream of nitrogen. Commercial linoleic acid was also subjected to alkaline isomerization following the same conditions.

FAs obtained after alkaline isomerization from both process were methylated to fatty acid methyl esters (FAME) and analyzed by gas chromatography (GC). Data showed that 24 and 48 hr of isomerization were sufficiently enough to produce a satisfactory amount of CLA from LA and SFA (75.74, 77.67%), respectively.

CLA produced after 24 hours of continuous isomerization of linoleic acid (75.74%) was examined for potential cytotoxicity by SRB assay. Results revealed that the prepared CLA exhibited an efficient cytotoxicity against breast carcinoma cell line (MCF7) with IC_{50} value of 2.42μg.

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INTRODUCTION

Conjugated linoleic acid (CLA) represents a family of conjugated polyunsaturated fatty acids with different positional and geometric double bonds. CLA is naturally present in animal products from ruminants; the main dietary sources (3-4mg/g in beef meat, 5-7mg/g in milk and butter) because of its formation as an intermediate of biohydrogenation by anaerobic bacteria in the rumen, but the presence of CLA was also verified in seed oils (0.01-0.12% of total fatty acids) (Cossignani et al., 2005). In addition, isomers of CLA are produced by free-radical induced isomerization of linoleic acid or during commercial hydrogenation of vegetable oils (Berdeaux et al., 1997).

CLA has been the subject of growing interest owing to its suspected nutritional and therapeutic properties including anticarcinogenic and anticholesterolemic properties and antiatherogenic actions. Since the first study by Pariza et al. (1979) showing an inhibitory effect of CLA on rat liver cells mutagen formation, many other studies demonstrated the beneficial effects of CLA isomers on human and animal health, such as reduction of mammalian or skin tumors (Ip et al., 1996) and decrease of breast or prostate cancer risk (Ochoa et al., 2004). It also appears that CLA formation may has some very advantageous nutritional properties, many authors found some beneficial effects of dietary CLA on lipid metabolism, such as body fat reduction with enhancement of lean body mass. Finally, CLA could be involved in immune function preservation, arthritis and inflammatory diseases or atherosclerosis in animals (Villeneuve et al., 2005).

Endogenous production of CLA isomers by humans is very limited. Therefore, a very large proportion of CLA found in the body tissues are from dietary origin (Villeneuve et al., 2005).

At present, the interest in CLA as commercial nutritional supplements or complements is tremendous and different products are now commercially available (Saebo, 2003). In most cases, these commercial products contain about 50 to 80% CLA. Various methods are available to produce synthetic CLA. The alkaline isomerization of linoleic acid is the most common. Generally, this reaction cannot be

carried out on natural linoleic vegetable oils such as sunflower, soy, or safflower, but instead must be produced from their corresponding soaps, which once conjugated through the action of a strong base, are then transformed into free fatty acids by dilute acid. The reaction temperature is around 200 to 250°C. Nowadays, alkaline isomerization of linoleic acid is often performed in propylene glycol in order to limit reaction temperature (<100°C) and catalyst quantities (2%) (Delmonte et al., 2004).

Linoleic acid is often produced during fat splitting industry and is commercially available in different purities.

More recently, new methods have been described in which transition metal catalysts are used that allow satisfactory CLA production.

Dehydration of castor bean oil is another well-known process that was extensively studied about 60 years ago (Larock et al., 2001). This oil contains about 85 to 90% ricinoleic acid, which can be easily dehydrated at high temperatures under acid catalysis to form an additional double bond in the aliphatic chain. Depending on the dehydration conditions, this newly formed double bond can be conjugated or not with the one initially present, leading to various CLA isomers and nonconjugated linoleic acid. It is worth noting that the main objective of these early publications was to obtain CLA-enriched TAG for painting and varnishing applications, not for the nutritional benefits of CLA isomers.

However, dehydration of ricinoleic acid still appears to be a good strategy to obtain CLA in satisfactory yields. For example, Berdeaux et al. (1997) showed that methyl ricinoleate could be transformed into the corresponding methylate, which was then reacted with a base to give a product containing 66% of CLA isomers. Recently, a similar process was applied by using potassium hydroxide to reduce the economic costs of the process and a 77% CLA was observed at 80°C (Yang et al., 2002).

In the present study, three different substrates were used to prepare CLA; dehydration of castor bean oil and isomerization of linoleic acid either separated from oil rich in linoleic or from linoleic acid.

MATERIALS AND METHODS

Materials:

Castor bean (*Ricinus communis*) was purchased from El-Sharkya Gavernerate.

Sunflower oil was purchased from El-Aiyat Factory, Cairo Oil and Soap Co., Giza, Egypt.

Commercial linoleic acid (~60%) was purchased from Sigma Chemical Co. (Saint Quentin, France).

Butylated hydroxy toluene (BHT) and all chemicals and solvents were purchased from Merck (Darmstadt, Germany).

Experimental :

Extraction of castor bean oil:

Oil was extracted from castor bean by using laboratory hydraulic press (Carver-type) under 10.000 metric tons pressure for 1h at room temperature. The extracted oil was filtered and kept in dark glass bottle (200 ml) at -18°C till analysis.

Dehydration of castor bean oil

Castor bean oil was dehydrated following the method described by Villeneuve et al., (2005); Oil (250 ml) was magnetically stirred (250 rpm) and heated in a 500 ml three neck round flask using a hot plate magnetic stirring (Jenway 1203Hoptplate Magnetic Stirrer) adjusted at the selected temperature in the presence of zinc powder (1g) as anti-polymerization agent. After 20 min equilibration at the selected temperature, phosphoric acid as a catalyst was added, and the dehydration reaction was then initiated. The reaction medium was maintained under vacuum to allow the removal of water formed during the process, and gentle bubbling with nitrogen was used to limit the presence of oxygen and possible oxidation reaction. Samples (50 mg) were taken periodically from the reaction medium using a syringe and transformed into FAME for GC analysis and determination of FA composition.

Dehydration process was also followed by the determination of conjugated diene (A.O.C.S. Methods, 1993), refractive index and iodine value (A.O.A.C., 2000).

Separation of FA from oil rich in linoleic acid

Sunflower oil (20g) rich in linoleic acid (62%) was reacted with 6.0g potassium hydroxide, 8.8 ml water, 53.2 ml 95% ethanol and 4mg butylated hydroxy toluene. The reaction was carried out for 1 h at 70°C, then the mixture was cooled and, after the addition of water, (40ml), it was washed with hexane, (2x50 ml). HCl (3N) was added to the aqueous phase to reached pH 1, then it was extracted with hexane, 2x50 ml, to isolate the free FAs (Cossignani et al., 2005). The organic layer was dried over anhydrous sodium sulfate, and then the FAs obtained rich in LA were subjected to isomerization reaction to obtain CLA.

Isomerization of fatty acids rich in linoleic acid

Commercial linoleic acid (~60%) with fatty acid composition of palmitic acid; 16:0 (13.2%), stearic acid; 18:0 (1.67%), oleic acid; 18:1 (31.73%), linoleic acid; 18:2 (52.00%) and linolenic acid; 18:3 (1.39%), and fatty acids fraction from sunflower oil rich in LA (~4g), were used as two different substrates for the isomerization process following the method described by Cossignani et al., (2005); 5ml n-butanol and 1g KOH were added and the reaction was heated to reflux at 120°±5°C under a gentle stream of nitrogen. Isomerization process was conducted in two different pathways; first, for 12 hr daily for 2 consecutive days, second, for continuously 48 h, every 12h, samples were drawn and after cooling, 5ml water was added to the reaction mixture, the mixture was acidified to pH 1 with 6N HCl and extracted with hexane, (2x10ml). The organic extracts were washed with 5% NaCl, (2x5 ml), and with water to reach neutrality, then dried over anhydrous sodium sulfate and evaporated under vacuum. A 50mg of samples were transformed into FAME for GC analysis for monitoring the formation of CLA.

Analytical Methods

Determination of fatty acids

Methylation of fatty acids:

An aliquot of fatty acids, (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 by centrifugation was 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).

GC analysis of FAME

Agilent 6890 series GC apparatus provided with a DB-23 column (60m x 0.32mm x 0.25μm). Fatty acids methyl esters were directly injected into the GC. Carrier gas was N_2 with a flow rate of 2.2 ml/min, splitting ratio of 1:80. The injector temperature was 250°C and that of FID detector was 270°C. The temperature settings were as follows: 150° to 225°C at 5°C/min, and then held at 225°C for 20 min (Villeneuve et al., 2005).

Moisture, oil content, Refractive index, acidity (% as ricinolic acid), peroxide values, iodine values and conjugated diene and triene contents of castor bean oil were determined according to A.O.A.C. Methods (2000).

Measurement of potential cytotoxicity by SRB assay

Potential cytotoxicity of CLA produced from linoleic acid (75.74%) after only 24 h of isommerization process was tested using the method of Skehan et al. (1990) in Cancer Institute, Cairo Univ., Egypt.

- Cells for breast (MCF7), colon (HCT), larynx (HEP2) or liver (HEPG2) cancer were plated in 96-multiwell plate (10^4 cells/well) for 24 before treatment with the CLA to allow attachment of cell to the wall of the plate.
- Different concentrations of CLA (0, 1, 2.5, 5 and 10μg/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose (concentration).
- Monolayer cells were incubated with the prepared CLA for 48 hours at 37°C in atmosphere of 5% CO_2.
- After 48 hr, cells were fixed, washed and stained with sulphorhodamine-B stain.
- Execs stain was washed with acetic acid and attached stained was recovered with Tris EDTA buffer.
- Color intensity was measured in an ELISA reader.
- The relation between surviving fraction and tested CLA concentration was plotted to get the survival curve of each tumor cell

line after the cytotoxicity of the specified compound and IC_{50} (dose of the tested CLA which reduces survival rate to 50%) were evaluated. Material that caused less than 50% survival was considered as anticancer agent for the organ it was tested for.

RESULTS AND DISCUSSION

Physicochemical properties of castor bean oil

Castor bean (4.07% moisture content) was ground and the oil was extracted by Carver hydraulic press. After oil filtration, physicochemical properties of the obtained oil were determined and the data are shown in Table 1. The obtained results revealed that, refractive index (RI), acidity (% as ricinolic acid), peroxide (PV) and iodine values (IV) were as follow; 1.4762, 1.59%, 0.00 and 87.39, respectively. Castor bean oil also had an absorbance at 232 and 270 nm of 0.22 and 0.31, respectively. Oil content (determined with Soxhlet apparatus) was 48.4%.

Table 1 : Physiochemical properties of castor bean oil

Parameters	Values
R.I. at 25°C	1.4762
Acidity (% as ricinoleic acid)	1.59
P.V. (meq peroxide/kg oil)	0.00
I.V. (Hanus)*	87.39
UV absorbance (E_{1cm} 1% at 232nm)	0.22
UV absorbance (E_{1cm} 1% at 270nm)	0.31

*Reported as numbers of grams of iodine required to saturate 100 grams of the sample

Dehydration of castor bean oil

Although several recent publications described the dehydration of ricinoleic acid as FFA or FAME (Berdeaux et al., 1997 and Yang et al., 2002). Direct dehydration of castor bean oil was more attractive since the recovered final products would be as TAG forms. These were found to be the most suitable forms for incorporation into functional foods and for bioavailability of CLA isomers in comparison with the alkyl ester forms (Fernie et al., 2004). In the presence of

catalyst and heat, castor bean oil can be easily dehydrated with good conjugation yields.

Table 2 : Changes in ricinoleic acid content (%) over the dehydration reaction of castor bean oil

Fatty acids%	Reaction Time (hours)						
	Zero	1	2	3	4	5	6
LA*	9.55	18.32	20.24	17.37	15.91	18.81	13.96
CLA** isomers	1.87	12.27	33.66	50.93	42.02	42.17	42.91
Ricinoleic acid	73.70	38.34	13.08	7.26	7.66	4.15	1.28

*LA : Linoleic acid

** CLA : Conjugated linoleic acid

CLA formation was followed by GC over the course of the dehydration reaction (Table 2). The dehydration reaction was very fast since residual ricinoleic acid was only 38.34% after the first hour. After 6 hours, dehydration was almost completed, as there was a minimal content (1.28%) of unreacted ricinoleic acid. However, the reaction produced not only conjugated isomers but also methylene - interrupted (nonconjugated) linoleic acid in high proportions. A considerable amount (50.93%)of CLA was produced within the first 3 h of the dehydration reaction. After 6 h of the reaction total CLA isomers were 42.91%. Table 2 shows that 3 h was sufficient to produce high yield of CLA isomers (50.93%) with a little amount of residual unreacted ricinoleic acid (7.26%) and prolongation of reaction time wasn't favored for the formation of CLA.

Changes that occurred during the dehydration process of castor oil were followed by monitoring changes in refractive index, iodine value and the formation of conjugated diene. Results in Table 3 clearly indicate that formation of conjugated isomers by the increase in conjugated diene reached its maximum after 3 of the reaction (78.28%).

Refractive index at 25°C and iodine value were continuously and progressively increased with the progress of reaction time (Table 3) and with the decrease of ricinoleic acid content (Table 2).

Table 3: Changes occurred during the dehydration process of castor bean oil

Reaction Time (hours)	% Conjugated diene	Iodine Value	Refractive Index
Zero	0.22	87.39	1.4762
1	28.25	93.57	1.4770
2	38.47	95.89	1.4790
3	78.28	113.26	1.4830
4	26.40	112.58	1.4840
5	23.40	117.83	1.4850
6	27.24	119.51	1.4870

Isomerization of fatty acids separated from sunflower oil

Sunflower oil rich in linoleic acid (LA) was chosen to study the effect of the isomerization reaction on LA content and the conversion into conjugated linoleic acid (CLA). Fatty acids (FAs) were first separated by alkaline saponification of sunflower oil and the obtained FAs were subjected to the isomerization reaction. Several trails (preliminary experiments) were conducted to reach the final CLA product which was followed by monitoring the changes in the LA content (data not shown). The results obtained from the final experiment are tabulated in Table 4 the results showed high content of LA in sunflower oil (62.08%), and that the formation of CLA isomers were very slow during the first 24 h and increased through the next 24 h reaching 12.22% after 36 h and the maximum formation was obtained after 48 h of the isomerization reaction under the experimental conditions (77.67%).

Table 4: Changes in linoleic and conjugated linoleic acids content (LA, CLA%) over the isomerization reaction course of fatty acids separated from sunflower oil

Fatty acids%	Reaction time (hours)				
	Zero	12	24	36	48
LA	62.08	49.74	53.23	52.11	11.89
CLA Isomers	--	7.47	5.77	12.22	77.67

Isomerization of commercial linoleic acid

Commercial linoleic acid (LA, 52.0%) was subjected to alkaline isomerization reaction at 120°±5°C for 12 h daily for 2 consecutive days. Samples were withdrawn at the end of the experimental day and analyzed by GC for monitoring the changes occurred in LA content. Results in Table 5 indicate that, under the experimental conditions there was a slow formation of CLA isomers reaching only 10.65% at the end of first day (12 h) and increased to 31.15% at the end of second day (24 h).

These results suggested the continuation of the process for 48 hours at 120°C.

Commercial linoleic acid was also subjected to the same conditions of the isomerization reaction tracing the formation of CLA isomers was followed (Table 6). The obtained FA profile of samples withdrawn every 12 h revealed a dramatic decrease in LA content from 52% at the beginning of the experiment reaching 9.58% through the first 24 h and that was accompanied with the increase in the formation of CLA reaching maximum content of 75.74% after only 24 h. Further reaction hours were accompanied by the conversion of CLA to nonconjugated linoleic acids. These results indicated that, prolongation of reaction time wasn't favor for the formation of CLA during the rest 24 h.

Table 5: Changes in linoleic and conjugated linoleic acids content (LA, CLA %) over the isomerization reaction course of linoleic acid

Fatty Acids %	Reaction Time (hours)*		
	Zero	12	24
LA	52.00	38.09	18.88
CLA isomers	--	10.65	31.15

* Alkaline isomerization reaction at 120°±5°C for 12 hr daily for two consecutive days

Table 6: Changes in linoleic and conjugated linoleic acids content (LA, CLA %) over the isomerization reaction course of linoleic acid

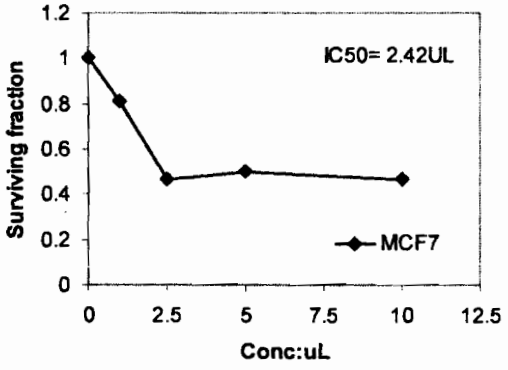
Fatty acids%	Reaction time (hours)*				
	Zero	12	24	36	48
LA	52.00	19.59	9.58	12.09	14.13
CLA isomers	--	41.24	75.74	55.79	60.95

* Isomerization process was carried out for continuously 48 hr

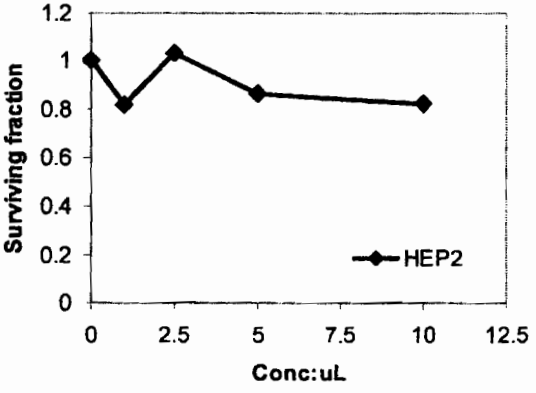
Biological evaluation

The obtained conjugated linoleic acid (CLA) with 75.74% through isommerization process of linoleic acid for 24 h was evaluated at the National Cancer Institute (Egypt) for cytotoxicity activity 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 reveal that CLA exhibited an efficient cytotoxicity against breast carcinoma cell line (MCF7) with IC_{50} value of 2.42μg (Fig 1-A). On the other hand CLA was proven to have no cytotoxic effect against the rest of carcinoma cell lines (Figs 1-B, C, D), respectively.

Although, the present data showed no anticancer effect of CLA against liver carcinoma cell line under the experiment conditions contrary to the results found by Pariza et al. (1979), several workers proved that CLA had an anticancer effect against breast carcinoma cell line (Ip et al., 1996; Lavillonniere and Bougnoux, 1999 and Ochoa et al., 2004)



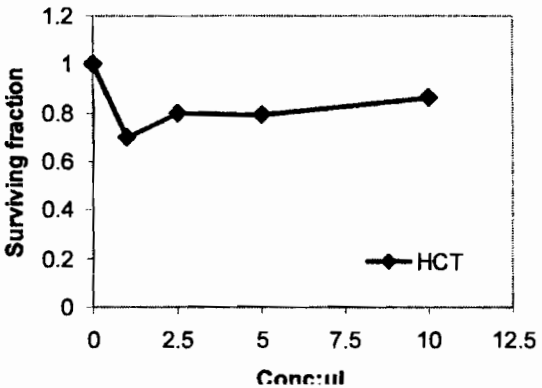
(A)



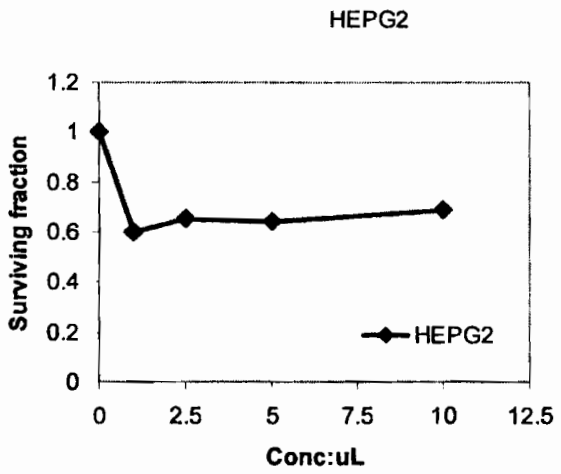
(B)

Fig 1 : Cytotoxic activity of CLA . (A) Against breast carcinomal cell line (MCF7) (B) Against colon carcinomal cell line (HCT)

Conjugated linoleic acid



(C)



(D)

Fig 1 : Cytotoxic activity of CLA . (C) Against larynx carcinomal cell line (HEP2) (D) Against liver carcinomal cell line (HEPG2)

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تحضير حامض اللينوليك فى الصورة المتبادلة وتقييمه بيولوجيا

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تم تحضير حامض اللينوليك فى الصورة المتبادلة بإستخدام ثلاث طرق مختلفة ١- تجفيف (نزع الرطوبة) زيت الخروع ٢- إجراء عملية تكوين المشابهات للإحماض الدهنية المنفصلة من الزيوت الغنية فى محتواها من حامض اللينوليك بعد تصبنها، ٣- إجراء عملية تكوين المشابهات لحامض اللينوليك التجارى.

تمت عملية نزع الرطوبة (التجفيف) على درجة حرارة ٢٥٠ ±٥ °م لعدة ساعات بإستخدام حامض الفوسفوريك (٠,١% وزن/ وزن) كعامل مساعد. وتم الحصول على نسبة عالية من حامض اللينوليك فى الصورة المتبادلة تصل الى ٥٠,٩٣% بعد ٣ ساعات فقط وذلك عن طريق تتبع التغيير فى الأحماض الدهنية وقياسات تحليلية اخري .

تم إجراء عملية تكوين المشابهات للاحماض الدهنية المفصولة من احد الزيوت الغنية فى محتواها من حامض اللينوليك باستخدام البوتاسا الكاوية على١٢٠±٥ °م لمدة ٤٨ ساعة وذلك فى وجود كحول البيوتانول تحت جو من النيتروجين . كذلك تم إجراء عملية تكوين المشابهات لحامض اللينوليك التجاري تحت نفس الظروف السابقة. واوضحت نتائج التحليل الكروماتوجرافى الغازى ان التفاعل لمدة ٢٤ ساعة اعطى نسبة عالية من حامض اللينوليك فى الصورة المتبادلة.

تم تقييم العينة المحضرة بعد إجراء عملية تكوين المشابهات لحامض اللينوليك لمدة ٢٤ ساعة متواصلة (٧٥,٧٤%) بيولوجيا وأثبتت النتائج ان لها تأثير واضح في وقف نشاط خلايا الثدي السرطانية بقيمة IC_{50} تبلغ ٢,٤٢ميكروجرام، بينما لم يكن لها تأثير في وقف نشاط الخلايا السرطانية للكبد والقولون والمرئ.