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The enrichment of sunflower oil with CLnA and antibacterial action

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Abstract: **Enzymatic acidolysis process was researched for the modification of sunflower oil by incorporation of CLnA (conjuge linolenic acid) isomers. Immobilized sn-1,3 specific lipase from *Mucor miechei* was used to modify sunflower oil to produce a new fatty acid chain with improved physico-chemical and functional properties. The acidolysis reaction of sunflower oil with pomegranate and mahlab seed oil, which is rich in CLnA, was actualized in the conditions with 10% enzyme, at 55°C, and in 1:1 substrate molar ratios. The incorporation ratio of CLnA into sunflower oil was established between 1.2% and 23.8% and so structured lipids were produced. Also, the antibacterial action of samples taken every 40 minutes from *E. coli* were researched. As a result, a strong antibacterial activity of CLnA extracted from structured sunflower oil was detected.**

Keywords: **CLnA (conjuge linolenic acid), Anti-*Escheichia coli*, Structured sunflower oil.**

I. INTRODUCTION

Structured or designed lipids are triacylglycerols that have been modified by the incorporation of new fatty acids or by changing the positions of existing fatty acids. By their functional properties, fat based foods with desired properties are produced. Especially fatty acids with high industrial importance such as conjugated fatty acids (CLA), oleic acid, linoleic acid, linolenic acid, stearidonic acid are used. In recent years, structural oil researches related to CLnA that prevent obesity, infectious diseases, heart disease, treat inflammation and are found to be extremely healthy have increased rapidly[1-3].

CLnA is a fatty acid group of positional and geometric isomers of octadecatrienoic acids that contain three double bonds in conjugation. CLnA isomers are known as *trans*-9,*trans*-12,*trans*-15-Octadecatrienoic acid, *trans*-9,*trans*-12,*cis*-15-Octadecatrienoic, *trans*-9,*cis*-12,*trans*-15-Octadecatrienoic, *cis*-9,*trans*-12,*trans*-15-Octadecatrienoic acid, *cis*-9,*cis*-12,*trans*-15-Octadecatrienoic acid, *cis*-9,*trans*-12,*cis*-15-Octadecatrienoic acid, *trans*-9,*cis*-12,*cis*-15-Octadecatrienoic acid, cis-9,cis-12,cis-15-Octadecatrienoic acid. CLnA commonly exists in seed oils of some plants such as pomegranate, marigold and mahlab[4,5]. The enzymatic process was an effective way to produce structured phospholipids with CLnA. Lipases offer a great advantage in catalyzed interesterification reactions due to their regiospecificity and selectivity. Therefore, Lipase-catalyzed reactions were used for the hydrolysis of triacylglycerols and the transesterification of triacylglycerols with fatty acids[6,7].

In our study, the enrichment of sunflower oil (*Helianthus annuus* L.) with CLnA from pomegranate (*Punica granatum* L.) and mahlab (*Prunus mahaleb* L.) seed oils and their antibacterial properties were researched. For this purpose, the acidolysis reaction of sunflower oil (SO) with pomegranate oil fatty acid (POFA) and mahlab oil (MOFA), which is rich in CLNA, were actualized in the presence of *Mucor miechei* lipase. The effects of the reaction time on incorporation yield and antibacterial condition were investigated.

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II. MATERIALS AND METHODS

A. Plants, Chemicals and lipases

Pomegranate and mahlab seeds used in this study were purchased from Göknur and Mecidefendi firms and their oils were extracted by the cold presser machine. All chemicals used were of chromatographic grade and antibacterial experiments were purchased from Sigma-Aldrich and Merck. Lipozyme IM (*Mucor miehei* lipase; MuLip) was purchased from Novo-Nordisk A/S (Copenhagen, Denmark). The cold presser were used to extraction of oil from pomegranate and mahlab seeds purchased Göknur and Mecidefendi firms.

B. Extraction of CLnA and Lipase-catalyzed acidolysis reaction

CLnA of pomegranate and mahlab oil were extracted as described previously in the works of Arboleda and Elibal works [4,8]. For this purpose, CLnA were isolated by saponification of pomegranate and mahlab oils. About 9 g of the seed oil was mixed with 90 mL 1 N KOH and heated at 90 C for 1 h. Then, 90 mL water was added to the mixture, and unsaponified components were obtained using hexane with separatory funnel. HCl (3N, 35 mL) was added to the soap solution and the free fatty acids (FFAs) were obtained with hexane. Finally, hexane was removed with a rotary evaporator and thus extracted pomegranate oil fatty acid (POFA)and mahlab oil (MOFA) were used for acidolysis reactions. The reaction mixtures consisted of 5 mL of n-hexane and a mixture of sunflower oil and CLnA; sunflower oil and pomegranate oil fatty acid (POFA); and sunflower oil and mahlab oil (MOFA) at 1:1 substrate molar ratios were placed in dark-coloured, heat resistant 20-mL reaction flasks. Lipozyme (MuLip; 10% wt of total substrates) was added to the reaction mixture. Reaction flasks were placed in an orbital shaking water bath at 250 rpm. All reactions were performed in 55^{o}C and samples of 2 ml were taken every 40 minutes. The enzymatic reaction in every sample was stopped by the addition of ethanol[2,9,10]. was extracted from producted structured oil as described previously in Arboleda and Elibal's works[4,8].

C.Fatty acid composition analysis

Sunflower oil and reaction products (CLnA components of extracted from structured oil) were converted into corresponding fatty acid methyl esters according to Jennings and Akoh's work[11]. The results were compared with CLnA (Sigma-Aldrich-supelco) and FAME (Sigma-Aldrich-supelco) standards studied in the same condition. The GC conditions were: Column: HP88, oven 200^{o}C, carrier gas: helium, 20 cm/sec, at 200^{o}C, FID detector: 250^{o}C, injection mode 1 µl, 220^{o}C, Split 100:1.

D. Anti- *Escherichia coli* screening of CLnA extracted from structured oils

Antibacterial activity of the structured oils were researched on *E. coli* (ATCC 25293) in every 40 minutes. Firstly, the bacterium inoculum was prepared in 4 ml-Triptic Soy Broth medium and incubated at 37^{o}C, overnight. After 24 hours, the culture suspensions were adjusted to 0.5 McFarland Standard Turbidity (~104) and stored at +4°C until use[12]. The Anti-*Escherichia coli* assay was carried out by the disc-diffusion to compare the antibacterial activities of the CLnA extracted structured oil. For the experiments; A certain amount of bacterium solution was spread on a solid agar medium in petri dishes (Nutrient agar) and filter paper discs (6 mm in diameter) were soaked in 20 µl of the CLnA and were placed on the inoculated plates. After the incubation at 37°C for 12 h, the diameters of the inhibition zones were measured in millimeter. Sterile distilled (20 µl) water was used as negative control and all tests were replicated three times[13,14].

III. RESULTS

Pomegranate and mahlab seed oils, as a natural source of CLnA, were used for remodification of sunflower oil using Lyposyme MuLip. The components of cold-pressed oil of sunflower oil were detected by GC. The results of the chemical composition of SO were presented in Table 1. It was reported that the main components of SO were linoleic acid 51.24%, oleic acid 36.29%, palmitic acid 7.38% and stearic acid 4.8%. No CLnA isomer was detected.

Table 1. The fatty acid composition of cold-pressed sunflower seeds

Fatty acid	%Area	Ret.time
C14:0 Myristic acid	0.1	19.962
C16:0 Palmitic acid	7.38	22.629

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C16:1 Palmitoleic acid	0.09	24.047
C18:0 Stearic acid	4.8	26.898
C18:1 Oleic acid	36.29	28.900
C18:2 Linoleic acid	51.24	32.143
CLnA isomers	-	-

The yield of oil cold-pressed sunflower oil, pomegranate and mahlab seeds were detected as 25%, 15%, 20%, respectively. The yield of CLnA extracted from pomegranate and mahlab were also determined as 3,96% and 22,46%, respectively (Table 2).

Table 2. The yield of oil and CLnA of cold-pressed sunflower, pomegranate and mahlab seeds

Yield %	sunflower	pomegranate	mahlab
Oil	25	15	20
CLnA	-	3,96	22,46

In this study, the enzymatic acidolysis method for the production of new modified fats was applied using immobilized lipase (MuLip). This enzyme catalyzed acidolysis reaction resulted in substantial replacement of long chan fatty acid residues in cold-pressed sunflower oil with CLnA extracted from PO and MO to produce new triglycerides which have significantly biological activity. We researched the effect of the reaction time on the acidolysis of SO with CLnA from PO and MO. At the optimum reaction temperature of MuLip, the acidolysis reactions were initiated and samples were collected every 40 minutes. In the present study, acidolysis reaction was observed resulting between 1.2% and 23.8% reduction of CLnA 1 isomer (trans-9,trans-12,trans-15-Octadecatrienoic acid) during 200 minute. The maximum incorporation of CLnA from pomegranate and mahlab seed oil were 12.1% and 9.9% after 120 min, respectively. In other words, CLnA isomer in SP and SM reached up to 12.1% and 9.9% (max) at the 120 min of reaction. After 40 min, in the 200 reaction time; 23.8% CLnA isomer in SC was observed (Table 3).

Table 3. The ration of CLnA in sutructured sunflower oil with CLnA (positive control): SC; pomegranate CLnA; SP and mahlab CLNA: SM in different time(min); 1/1 mol/mol; 55°C, %10 MuLip.

CLnA isomers/time	**0**	**40**	**80**	**120**	**160**	**200**
trans-9,*trans*-12,*trans*-15-Octadecatrienoic acid	SC:9.8 SP:6.3 SM:6.6	SC:4.5 SP:4.9 SM:1.8	SC:1.2 SP:4.2 SM:1.2	SC:4.07 SP:12.1 SM:9.9	SC:23.8 SP:7.6 SM:4.9	SC:1.7 SP:1.7 SM:1.6

Strong antibacterial activity were noticed for mahlab (between 2.0-2.81 mm) and pomegranate (between 1.19-2.86 mm) at 0, 40, 80, 120, 160, 200 minutes. Most strong antibacterial activity was shown for Mahlab (2.81 mm), pomegranate (2.86 m) at 120 minutes (Table 4).

Table 4. Anti-*Escherihia coli* activity of CLnA isomer (*trans*-9,*trans*-12,*trans*-15-Octadecatrienoic acid) in sutructured sunflower oil with CLnA (positive control): SC; pomegranate CLnA: SP and mahlab CLnA: SM. Inhibition zone on disc diffusion method (mm)

Min.	**0**	**40**	**80**	**120**	**160**	**200**
SC	2.47±0.01	2.17±0.05	2.20±0.13	2.20±0.01	3.13±0.02	2.27±0.04
SP	2.57±0.19	1.19±0.15	2.47±0.01	2.86±0.09	2.50±0.01	2.27±0.03
SM	2.78±0.07	2.0±0.11	2.20±0.02	2.81±0.001	2.57±0.02	2.33±0.02

In other words, at the end of 120 minutes, the CLnA rate reached the maximum level either in acidolysis (Figure 1a) and antimicrobial effect graph (Figure 1b). Therefore, it was determined that this isomer inhibited the growth of microbial cells.

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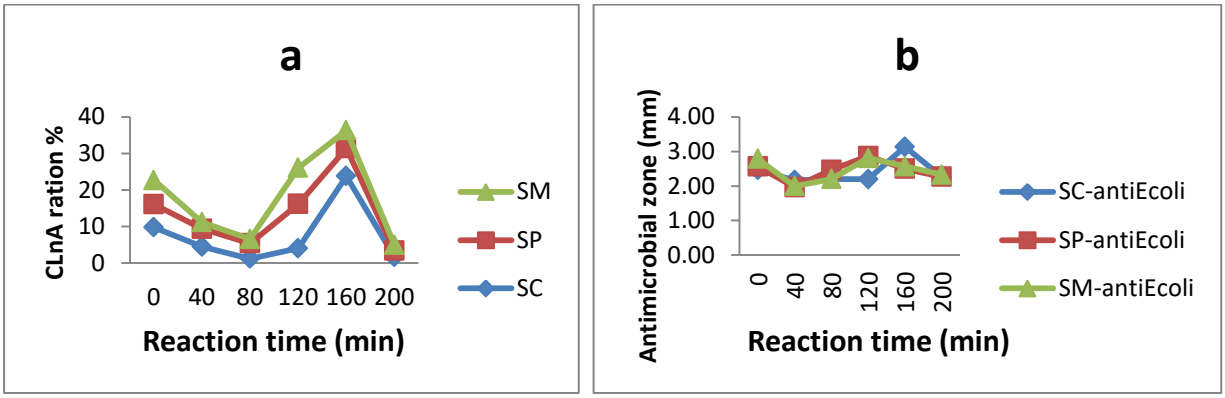

Fig. 1. (a) Effect of reaction time on the Lipozyme™-catalyzed interesterification of sunflower oil (SO) with CLnAs positive control (1/1 mol/mol; 55°C, %10 MuLip). (b) Anti-*E. coli* activity of CLnAs extracted from sutructud sunflower oil (SO).

IV. DISCUSSION

The studies in literature demonstrated that temperature is one of the important parameters on the efficiency of lipase enzyme by affecting its catalytic activity [15]. According to literature, the working temperature of the Lipozym RM IM (*Mucor* sp. lipase) is about 50 degrees. In addition, previous studies demonstrated that the reaction time is ranging from 6h to 72 h for acidolysis of immobilized lipases such as Lipozym RM IM, and Lipozym TL IM [7,16]. In the present study, an acidolysis reaction occured which could be attributed to the effect of MuLip resulting in max. 23.8% of CLnA in 55°C and 160 minutes. Süzen and coworkers detected the incorporation ratio of conjugated linolenic acid as %41,4 in the study about the enrichment of corn oil by enzymatic reaction with CLnA extracted from bitter [17]. In another study, the maximum incorporation of CLnA from pomegranate seed oil and ICM from flaxseed oil into phosphotidylcholine (PC) using Liposyme TL IM, was 11.3% and 4.9% after 72 h, respectively[8]. Elibal and coworkers were determined that the incorporation ratio of CLnA into olive oil was %41 in the optimum reaction conditions (substrate molar ratio of 1:3,5 of Olive oil: Pomegranate fatty acid, %9,7 enzyme and 60°C temperature) [4].

The chromatograms from the GC analyses were visually compared for the presence or absence of any CLnA isomers in structured sunflower oil. Under these condition, CLnA isomers known as *trans*-9,*trans*-12,*trans*-15-Octadecatrienoic acid were produced in both SP and SM.

Conjugated linolenic acids have shown important health benefits such as antioxidant effects [18], anticarcinogenic [19], hypoglycemic [20] and antimicrobial [21]. In this work, it has been shown that both CLnA extraction products obtained from structured oils have inhibitory effects on *Escherichia coli* strain.

V. CONCLUSION

In this study, modified sunflower oil was built up using pomegranate and mahlab seed oils which have enough CLnA. By using these plants, we produced only one CLnA isomer in sunflower oil and detected its strong *anti-E. coli* activity. Our work is an example in terms of the development of modified oils and therefore, this work is an example for further studies in this field.

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