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Full Length Research Paper

Antibacterial activity and cytotoxicity of the leaf essential oil of *Morus rotunbiloba* Koidz

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Essential oils from the leaf of *Morus rotunbiloba* Koidz was extracted and analysed using steam distillation and GC-MS. The results obtained indicated that the main components of the extracts are benzyl alcohol (10.48%), dihydroactinolide (10.11%) and palmitic acid (7.01%). The oil possessed strong antibacterial activity against *Vibrio cholerae* O139 DMST 9701, *Samonella paratyphi* A DMST 8486, *Serratia marcescens* ATCC 8100 and *Samonella choleraesuis* ATCC 10708 with minimum inhibition concentration of 1.33, 2.66, 2.66 and 10.65 µg/ml, respectively. The oil was tested for anticancer activity on human larynx epidermoid carcinoma and colon adenocarcinoma cell lines and found to have cytotoxic activity to both cancerous cell lines, with LC_{50} of 70 and 120 µg/ml, respectively. The results obtained from antibacterial and anticancer tests indicated that essential oils from *M. rotunbiloba* can be used for the applications in food and pharmaceutical proposes.

Key words: *Morus rotunbiloba,* essential oil, antibacterial activity, cytotoxicity.

INTRODUCTION

Plant extracts and their essential oils are interesting as sources of natural products for decades (Burt, 2004). Many kinds of essential oils have been screened for their potential uses for food preservation, aromatherapy and fragrance industry (Bakkali et al., 2008). Their alternative applications for the treatment of infectious diseases have also been studied. Some of them have been found to have antioxidant, antimicrobial, antigenotoxic and insecticidal properties (Tepe et al., 2005; Lee and Shibamoto, 2002; Pavithra et al., 2009; Burt, 2004; Bassole et al., 2003; Shukla et al., 2009; Giordani et al., 2006; Allahverdiyev et al., 2004; Schnitzler et al., 2001; Peres et al., 2009; Anthony et al., 2005; Singh et al., 2009; Franzios et al, 1997; Cheng et al., 2009). Also, others are found to have antimutagenicity and anticancer properties (Jayaprakasha et al., 2002; Peres et al., 2009; Kumar et al., 2004; Sylvestre et al., 2006). *M. rotunbiloba* and the other mulberry plants of *Morus* species are widely cultivated in Thailand and in many Asian countries. In Thailand, besides the usual use for feeding silkworm (*Bombyx mori* L.), its dried leaves have been consumed as herb-tea beverage and food supplements. Moreover, the plants have a long history in the Oriental medicine for anti-inflammatory, diuretic, antitussive and antipyretic purposes (Asano et al., 2001). *M. alba* L., *M. indica* L. and *M. rotunbiloba* have been reported to exhibit antioxidant and antihyperglycemic activity (Zhishen et al., 1999; Patrakorn et al., 2006; Andallu et al., 2001; Andallu and Varadacharyulu, 2003). *M. alba* L. and *M. mongolica* have been reported to have anti-viral activity, antibacterial activities and cytotoxicity against a cancer cell line, HepG2 (Du et al., 2003; Shi et al., 2001; Shon et al., 2004). So far, the study of those biological activities has been carried out in organic-solvent-extracts. No report on biological activities of the mulberry essential oil has been published. The objectives of this study were to identify the chemical compositions of the essential oil extracted from the leaf of *M. rotunbiloba* Koidz and to investigate its antibacterial and cytotoxic properties

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Table 1. Human-pathogenic bacteria used for antibacterial assay.

Acinetobacter anitratus DMST 4683	*Pseudomonas putida* DMST 0905
Acinetobacter lwoffii ATCC 15309	*Salmonella choleraesuis* ATCC 10708
Aeromonas hydrophila DMST 2793	*Salmonella enteritidis* DMST 8536
Alcaligenes faecalis ATCC 35655	*Salmonella paratyphi* A DMST 8486
Corynebacterium diphtheriae DMST 7471	*Salmonella typhi* DMST 5784
Bacillus cereus ATCC 11778	*Salmonella typhimurium* ATCC 13311
Burkholderia cepacia ATCC 2546	*Serratia marcescens* ATCC 8100
Enterobacter cloacae DMST 3557	*Shigella boydii* DMST 2982
Escherichia coli O157 DMST 4554	*Shigella boydii* DMST 7776
Escherichia coli group I (O119) DMST 10674	*Shigella dysenteriae* DMST 7123
Escherichia coli group II (O86) DMST 10693	*Shigella flexneri* ATCC 8100
Escherichia coli group III (O28) DMST 10695	*Shigella sonnei* (group D) DMST 2982
Escherichia coli ATCC 25922	*Streptococcus agalactiae* DMST 5071
Enterococcus faecalis ATCC 33186	*Staphylococcus aureus* ATCC 25923
Klebsiella pneumoniae ATCC 700603	*Staphylococcus epidermidis* DMST 559
Micrococcus varians DMST 5801	*Streptococcus pyogenes* DMST 3393
Moraxella osloensis DMST 4357	*Vibrio cholerae* non O1, non O139 DMST 2873
Pseudomonas aeruginosa ATCC 10145	*Vibrio cholerae* O139 DMST 9701
Pseudomonas aeruginosa ATCC 27853	*Vibrio cholerae* O1 serotype Ogawa DMST 9700
Pseudomonas fluorescens DMST 4597	*Vibrio minicus* DMST 8074
Proteus mirabilis ATCC 21100	

against human larynx epidermoid carcinoma and colon adenocarcinoma cell lines.

MATERIALS AND METHODS

Mulberry leaf essential oil

Mulberry leaf essential oil was extracted from fresh leaves of silkworm-mulberry, *Morus rotunbiloba* Koidz (locally named Mon-Noi) that were kindly provided from Udorn-Thani Sericulture Research Center, Thailand. The leaves were dried at 40°C and then finely ground in a mill and stored in tightly closed plastic bag at 4°C. Twelve grams were extracted continuously for 3 h using modified Clevenger-type steam distillation apparatus with 400 ml of water filled in the sample flask and 50 ml of dichloromethane filled in the other flask. The essential oil obtained in dichloromethane was dried over anhydrous sodium sulfate, evaporized and dissolved in 2 ml of dimethyl sulfoxide (DMSO, Sigma).

Analysis of mulberry leaf essential oil

Volatile compounds in the essential oil were analyzed using Hewlett-Packard GC-MS system on GC 5890 series II, equipped with mass selective detector (MSD 5972) and HP-5 MS (cross linked 5% phenyl methyl siloxane) capillary column (30 m × 0.25 mm, film thickness 0.25 μm). Injector and detector temperatures were set at 250 and 280°C, respectively. Oven temperature was kept at 60°C for 5 min, then gradually raised to 150°C at 5°C/min, for 5 min and then raised to 180°C at 5°C/min, for 5 min and finally raised to 200°C at 5°C/min. Helium served as carrier gas, at a flow rate of 1 ml/min. 1 μl of samples diluted at 1/200 dichloromethane (v/v) were injected in the splitless mode. For detection by MS, an electron ionization system with ionization energy of 70 eV was used. The components were identified by comparison of their relative retention times and mass spectra with the standards in Wiley 275 library data of the GC/MS system.

Antibacterial activity assay

Bacterial strains

The activities of essential oil were determined against forty-one strains of human-pathogenic bacteria as shown in Table 1. All of these bacteria were kindly supported by the National Institute of Health, Ministry of Public Health, Thailand and the Department of Biotechnology, Faculty of Science, Ramkhamhaeng University.

Agar disc diffusion method

The essential oil was preliminary screened for antibacterial activity by agar disc diffusion method (Bauer et al., 1966). The 18 h bacterial culture in Mueller-Hinton broth was diluted to approximately 10^5 CFU/ml with 0.85% normal saline solution. Five hundred μl of the suspension were spread over the Muller-Hinton agar plates. The essential oil was dissolved in 10% aqueous dimethylsulfoxide (DMSO) and sterilized by filtration through a 0.45 μm membrane filter. Empty sterilized paper discs (12 mm dia.) were impregnated with 100 μl of different concentrations of the essential oil and placed on the agar surface. Two sets of discs were performed for each organism. Paper disc moistened with aqueous DMSO was used as a negative control. Ampicillin, chloramphenicol and amphotericin B were used as positive control drugs. All plates were sealed with Para film to avoid the test samples evaporation and incubated at 37°C for 18 h. The inhibition zone was measured and the mean value was calculated.

Agar dilution method

Minimal inhibition concentration (MIC) values of the essential oil were determined by an agar dilution method (Barry, 1976; National Committee for Clinical Laboratory Standards, 1983). The two-fold dilutions of essential oils in DMSO were mixed with the sterile melted Muller-Hinton agar to the final volume of 20 ml and immediately poured into petri dishes. Ten microliters of 10^4 CFU/ml of each bacterium were spotted onto the plates and the plates were then incubated at 37°C for 24 h. The experiments were performed thrice and the inhibition of the bacterial growth was compared with the growth in the control plate. The MIC was defined as the lowest concentration of the oil inhibiting the visible growth of each organism on the agar plate. *Staphylococcus aureus* ATCC25923 and *Escherichia coli* ATCC25922 were used as standard reference strains.

Assay of cytotoxicity to cancerous cell lines

Cell lines and cell culture medium

A normal cell line, African green monkey kidney (Vero) cell, and two cancerous cell lines namely, human larynx epidermoid carcinoma (Hep2) cell and human colon adenocarcinoma (SW620) cell were used in this study. All cell lines were cultured in RPMI 1640 medium (Sigma), supplemented with 10% inactivated fetal bovine serum (FBS) (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml). The medium was then sterilized by filtering through 0.22 μm microbiological filters and kept at 4°C before use. The cell lines were maintained in a humidified atmosphere of 5% CO_2, -95% air at 37°C.

In vitro cytotoxicity assay

The cytotoxic effects of the mulberry leaf essential oil against tumor and normal cell lines were determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphynyltetrazolium bromide] colorimetric assay (Ferrari et al., 1990). Cell lines at the logarithmic growth phase were washed once with phosphate buffered saline (PBS), detached with trypsin ethylenediamine tetraacetic acid (Gibco), and resuspended to 5 x 10^4 cells/ml in fresh RPMI medium supplemented with 10% FBS. The cell suspension (1 x 10^4 cells) was placed in a 96-well flat-bottomed tissue culture plate. After the cells reached logarithmic growth phase, the supernatant was discarded. Two hundred microlitters of each concentration of essential oil, 0.1, 1, 10, 100, 200 and 400 μg/ml diluted in RPMI 1640 medium, supplemented with 10% FBS, were added into the plate. After 24 h incubation at 37°C, cell viability was evaluated. Briefly, the supernatants were removed from the wells and 200 μl MTT (Sigma) solution (2 mg/ml in PBS) was replaced. The plates were incubated for 2 h at 37°C, and 125 μl of DMSO was added to the wells to dissolve the MTT crystals. The plates were placed on a shaker for 15 min and the absorbance at 595 nm was read by a multi-well spectrophotometer. Cells treated with a cytotoxic drug, mitomycin C (0.5 μg/ml) were used as a positive control, whereas untreated cells were used as a negative control. The percentage of cytotoxicity was calculated as following:

% Cytotoxicity = 1- [(OD extract treated –OD blank)/ (OD control-OD blank)] x100

RESULTS AND DISCUSSION

Steam distillation of *M. rotunbiloba* dry leaf produced a pale-green essential oil with the average yield of 44.5%. GC-MS analysis of the oil sample led to the identification of 51 different compounds, as listed in Table 2, representing 76.31% of the total oil. As far as our literature survey, this is the first document reporting the chemical compositions of *M. rotunbiloba* leaf essential oil. Three major components were benzyl alcohol (α-toluenol, 10.48%), dihydroactinolide (10.11%), and palmitic acid (7.01%). The minor compounds were 2-hexenal (4.49 %), ethylmethyl-maleic anhydride (3.50%), 2,4-heptadienal (3.31%), caproic acid (3.19%) and ethylmethyl-maleimide (2.63%). Other components present less than 2% of the total yield. As shown in Table 3, the essential oil contained acids and derivatives as the highest part (31.02%), the other contents were aldehydes and ketones (18.63%) and alcohols (11.85%). Di-nor-sesquiterpenes and di-nor-sesquiterpenoids, hydrocarbons and miscellaneous, monoterpenes and monoterpenoids, phenolic compounds, and diterpene and dinor-diterpenoid were found in relatively low amounts of 4.74, 3.61, 3.22, 2.02 and 1.22% respectively.

The antibacterial activities of the essential oil were determined against forty-one strains of human-pathogenic and two strains of non-pathogenic bacteria. As shown in Table 4, the oil possesses strong antimicrobial activities against four gram-negative bacteria, *V. cholerae* O139 DMST 9701, *S. paratyphi* A DMST 8486, *S. marcescens* ATCC 8100 and *S. choleraesuis* ATCC 10708 with their respective MIC value of 1.33, 2.66, 2.66 and 10.65 μg/ml. Antibacterial activities of several essential oils against both gram-positive and gram-negative bacteria have been published (Burt, 2004). Organic-solvent-extracts of leaves and roots of other *Morus* species have been previously reported to be rich sources of flavonoids and other polyphenolic compounds which possess antioxidant and broad-spectrum antibacterial properties (Zhishen et al., 1999; Asano et al., 2001; Doi et al., 2001). In *Thymus* essential oils, the phenolic components were suggested to have antibacterial properties (Cosentino et al., 1999). A number of major compositions such as α-terpineol, citral, thymol, carvacrol, eugenol, geraniol and perillaldehyde in some plants essential oil have been proved to be effective against food-borne bacterial pathogens (Consentino et al., 1999; Kim et al., 1995). So far, some strains of *V. cholerae* showed susceptiblity to borneol, camphor, *trans*-caryophyllene and linalool, the major constituents of phenolic compounds found in essential oil of *Lepechinia caulescens*. These compounds were proposed to be antibacterial active agents (Acevedo et al., 2005). However, relatively low content of phenolic compounds and actively inhibited only some gram-negative bacteria, particularly to *V. cholerae* O139 DMST 9701 were found in our essential oil. Larynx epidermoid carcinoma and colon adenocarcinoma were two of the ten cancers often found in Thai patients. Two cancerous cell lines, human larynx epidermoid carcinoma (Hep2)

Table 2. Chemical constituents of *M. rotunbiloba* leaf essential oil analyzed by GC-MS.

Peak no.	Compounds	RT (min)	% Area
1	2-Hexenal	4.47	4.49
2	(R)-1-Methyl-2-cyclohexen-1-ol	7.58	0.40
3	Benzaldehyde	7.76	0.42
4	γ-Lactone	8.16	0.53
5	Methylheptenone	8.73	1.58
6	2,4-Heptadienal	9.53	3.31
7	Caproic acid	10.15	3.19
8	Benzyl alcohol	10.45	10.48
9	α-Phynyl acetaldehyde	10.64	1.02
10	2-Hexanoic acid	11.44	0.82
11	3,5-Octadiene-2-one	11.62	1.98
12	α,α-Dimethyl benzenemethanol	12.07	0.85
13	Ethylmethyl maleic anhydride	12.71	3.50
14	4-Ketoisophorone	13.93	0.29
15	Propiophenone	14.56	0.72
16	Naphthalene	15.08	0.64
17	Azulene	15.10	0.68
18	Safranal	15.64	1.69
19	5- Imidazolecarboxaldehyde	15.71	0.54
20	2-Hydroxy-3-propyl-2-cyclopenten-1-one	16.01	1.82
21	ß-Cyclocitral	16.27	1.94
22	α-Terpinene	16.47	0.57
23	Ethylmethyl-maleimide	16.83	2.63
24	α-Ionene	17.16	0.68
25	5-Penthyl-2(3H)-furanone	17.54	0.49
26	α-Citral	17.69	0.71
27	4-Vinyl-2-methoxyphenol	18.92	1.58
28	2-Bromonitrobenzene	19.47	0.64
29	γ-Heptalactone	20.23	0.25
30	Damascenone	20.82	0.19
31	7-Methoxy-2,2-dimethyl-2H-1-benzothione	21.21	0.32
32	(E)-α-Ionone	21.93	0.41
33	Geranyl acetone	22.55	1.41
34	Cyclododecane	23.04	0.42
35	2,4-Di-tert-butylphenol	24.19	0.44
36	Dihydroactinolide	24.88	10.11
37	2,3,4-Trimethyl quinoline	25.31	0.42
38	Megasticmatrienone 4	25.68	1.17
39	(E,E)-Pseudoionone	26.46	0.67
40	Diethyl phthalate	26.79	0.69
41	3-Keto-ß-ionone	29.76	0.21
42	Myristyl alcohol	29.93	0.12
43	9,-10 Dihydro-9,9-dimethylanthracene	30.53	0.34
44	2,3,6-Trimethyl-1,4 napthoquinone	32.66	0.28
45	Phenanthrene	33.04	0.15
46	Hexahydrofarnesyl acetone	35.17	0.17
47	Methylpalmitate	38.03	0.13
48	Butyl phthalate	39.50	1.98
49	Palmitic acid	39.53	7.01
50	Methyl linolenate	44.17	0.18
51	Trans-phytol	44.58	1.05
	Total		76.31

Table 3. Groups of compounds in the *M. rotunbiloba* leaf essential oil.

Compound	% Area	Compound	% Area
Acids and derivatives		**Aldehydes and ketones**	
γ-Lactone	0.53	2-Hexenal	4.49
Caproic acid	3.19	Benzaldehyde	0.42
2-Hexanoic acid	0.82	Methylheptenone	1.58
Ethylmethyl maleic anhydride	3.50	2,4-Heptadienal	3.31
Ethylmetylmaleimide	2.63	α-Phenyl acetaldehyde	1.02
γ-Heptalactone	0.25	3,5-Octadiene-2-one	1.98
Dihydroactinolide	10.11	Ketoisophorone	0.29
Diethyl phthalate	0.69	Propiophenone	0.72
Methyl palmitate	0.13	Safranal	1.69
Dibutylphthalate	1.98	5-Imidazolecarboxaldehyde	0.54
Palmitic acid	7.01	2-Hydroxy-3-propyl-2-cyclopenten-1-one	1.82
Methyl linolenate	0.18	5-Pentyl-2(3H)-furanone	0.49
Total	31.02	2,3,6-Trimethyl-1,4 napthoquinone	0.28
		Total	18.63
Alcohols			
(R)-1-methyl-2-cyclohexen-1-ol	0.40	**Monoterpenes and monoterpenoids**	
Benzyl alcohol	10.48	β-Cyclocitral	1.94
α-Cumyl alcohol	0.85	α-Terpinene	0.57
Meristyl alcohol	0.12	α-Citral	0.71
Total	11.85	Total	3.22
Hydrocarbons and miscellaneous		**Dinor-sesquiterpenes and Dinor-sesquiterpenoids**	
Azulene	0.68	α-Ionene	0.68
2-Bromonitrobenzene	0.64	Damascenone	0.19
7-Methoxy-2,2-dimethyl-2H-1- benzothiopyran	0.32	(E)-α-Ionone	0.41
Cyclododecane	0.42	Geranyl acetone	1.41
2,3,4-Trimethylquinoline	0.42	Megastigmatrienone	1.17
9,-10 Dihydro-9,9-dimethylanthracene	0.34	(E,E)-Pseudoionone	0.67
Phenanthrene	0.15	3-Keto-β-ionone	0.21
Napthalene	0.64	Total	4.74
Total	3.61		
Phenolic compounds		**Diterpene and dinor-diterpenoid**	
4-Vinyl-2-methoxyphenol	1.58	Hexahydrofarnesylacetone	0.17
2,4-Di-tert-butylphenol	0.44	Trans-phytol	1.05
Total	2.02	Total	**1.**22

Table 4. Minimum inhibitory concentration of *M. rotunbiloba* leaf essential oil against human pathogenic bacteria.

Pathogenic bacteria	Minimum inhibitory concentration (µg/ml)
Salmonella choleraesuis ATCC 10708	10.65
Salmonella paratyphi A DMST 8486	2.66
Serratia marcescens ATCC 8100	2.66
Vibrio cholerae O139 DMST 9701	1.33

Table 5. *In vitro* cytotoxicity of *M. rotunbiloba* leaf essential oil on Vero, Hep2 and SW620 cell lines.

Cell line	Concentration (µg/ml)	Cytotoxicity (%)		
		Vero cell	Hep2 cell	SW620 cell
Essential oil	0.1	0	2 ± 0.2	5 ± 0.1
	1	0	1 ± 0.1	5 ± 0.1
	10	0	8 ± 0.3	10 ± 0.5
	100	0	54 ± 1.9	39 ± 5.6
	200	26 ± 1.5	65 ± 1.7	93 ± 4.3
	400	68 ± 1.8	67 ± 2.5	NT
Mitomycin C	0.5	78 ± 0.1	NT	NT

NT, not tested; values are mean ± SD (n = 8).

and human colon adenocarcinoma (SW620) cell lines were used to determine anticancer properties of *M. rotunbiloba* leaf essential oil, and African green monkey kidney (Vero) cell line, a normal cell line was used as a control. All cell lines were treated with different concentrations of the *M. rotunbiloba* leaf essential oil for 24 h. The percentages of cytotoxicity were summarized in Table 5. Mitomycin C, the positive cytotoxic control agent, provided 78% cytotoxicity at very low concentration, 0.5 µg/ml. As shown in Figure 1, the oil at 0.1 - 100 µg/ml had no effect on Vero cell viability. The toxicity of the oil towards both human cancer cell lines was a dose dependent manner. The median lethal concentration (LC_{50}) of the oil on the cytotoxicity of Hep2, SW620 and Vero were 70, 120, and 280 µg/ml respectively. Other essential oils have been reported to have anticarcinoma activities, such as the leaf essential oils of *Croton flavens* L that have against human lung carcinoma cell line (A549) and human colon adenocarcinoma cell lines (DLD-1) at GI_{50} of 27 ± 4 and 28 ± 3 µg/ml, respectively, (Sylvestre et al., 2006).

Some monoterpenes and sesquiterpenes including α-cadinol, β-elemene, α-humulene (Sylvestre et al., 2005; Legault et al., 2003) and isoprenoids including geraniol and farnesol (Burke et al., 1997) were reported to be active against the tumor cell lines. However, a small amount of these compounds were found in our *M. rotunbiloba* leaf essential oil (Table 3). Although the major components of the essential oils generally represent the biologically features of the oils (Burt, 2004; Bakkali et al., 2008), they are not necessarily too responsible for the greatest activities (Lis-Balchin et al., 1998). Various minor components may contribute tobiological activities, possibly by synergistic effect between the components. This has been found in the case of essential oils of mint (Franzios et al., 1997), of certain species of *Thymus* (Marino et al., 1999) and of essential oils of sage (Marino et al., 2001). In the concept of synergism, it would be more meaningful to study biological activities of the entire oil rather than some parts of its components. In conclusion, the essential oil from *M. rotunbiloba* leaf contained a complex mixture of chemical

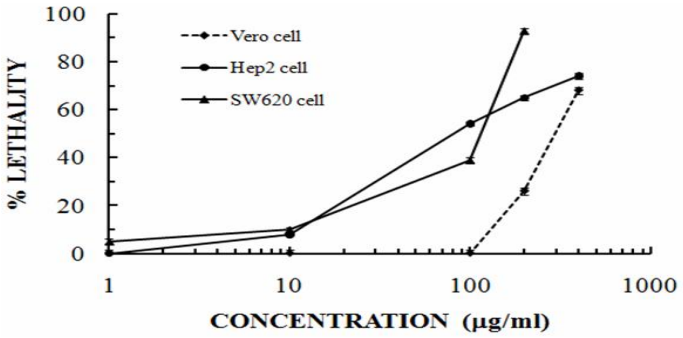

Figure 1. Cytotoxicity of *M. rotunbiloba* leaf essential oil African green monkey kidney (Vero), human larynx epidermoid carcinoma (Hep 2) and human colon adenocarcinoma (SW620) cell lines in the presence and absence of the essential oil were tested by means of an MTT assay. The values represented are means from two separated experiments, each performed in quartet.

substances. It exhibited antibacterial activities to four gram-negative bacteria with the strongest inhibitory to *V. cholerae* O139 DMST 9701. Moreover, the oil showed *in vitro* cytotoxicity against human larynx epidermoid carcinoma and colon adenocarcinoma cell lines. The antibacterial and anticancer activities of *M. rotunbiloba* leaf essential oil make an interest for the applications in food and pharmaceutical proposes. Before using as therapeutic purposes in human, the oil must be evaluated for safety and toxicity doses.

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