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ORIGINAL RESEARCH

Enhanced Cosmeceutical Potentials of the Oil from *Gryllus bimaculatus* de Geer by Nanoemulsions

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Purpose: This study aimed to extract the oil from *Gryllus bimaculatus* de Geer, evaluate its potential for cosmeceutical applications, and develop nanoemulsions to promote the cosmeceutical capabilities of the oil.

Methods: *G. bimaculatus* oil was produced by the cold pressing method. Its fatty acid compositions were assessed by fatty acid methyl ester/gas chromatographic-mass spectrometry. The antioxidant activities of the oil were investigated in terms of radical scavengers, reducing power, and lipid peroxidation inhibition. The whitening effects were investigated through anti-tyrosinase activities, whilst the anti-aging effects were investigated through inhibition against collagenase, elastase, and hyaluronidase. The irritant effects were investigated by the hen's egg chorio-allantoic membrane test and the cytotoxicity assay in immortalized human epidermal keratinocytes and human foreskin fibroblast cells. The nanoemulsions were developed, characterized, and evaluated for their stability and cosmeceutical properties.

Results: *G. bimaculatus* oil, rich in linoleic acid (31.08 ± 0.00%), oleic acid (30.44 ± 0.01%), palmitic acid (24.80 ± 0.01%), and stearic acid (7.61 ± 0.00%), demonstrated promising cosmeceutical properties in terms of antioxidant, anti-tyrosinase, and anti-skin ageing activities. Besides, the oil was safe since it induced no irritation or cytotoxicity. *G. bimaculatus* oil was successfully developed into nanoemulsions, and F1, composed of 1% w/w *G. bimaculatus* oil, 1.12% w/w polysorbate 80, 0.88% w/w sorbitan oleate, and 97% w/w DI water, had the smallest internal droplet size (53.8 ± 0.6 nm), the narrowest polydispersity index (0.129 ± 0.010), and a pronounced zeta potential (−28.23 ± 2.32 mV). All cosmeceutical activities of the oil were significantly enhanced after incorporation in the nanoemulsions ($p < 0.001$), particularly the whitening effects.

Conclusion: *G. bimaculatus* oil nanoemulsion was an attractive cosmeceutical formulation with potent whitening effects, along with antioxidant and anti-aging properties. Therefore, nanoemulsion technology was found to be an effective strategy for improving the cosmeceutical properties of *G. bimaculatus* oil.

Keywords: cricket, antioxidant, whitening, anti-skin ageing, required hydrophilic–lipophilic balance, high-pressure homogenization

Introduction

Nowadays, insects can be produced in large quantities and used both directly for human consumption and indirectly as livestock feed to produce animal proteins.[1] Insects have received attention and have been regarded as "green livestock" which are entirely consumed and produce no waste. Besides, edible insects have the potential to replace traditional animal- and plant-based nutrients in a healthy and sustainable manner due to their nutrient-rich composition.[2] Numerous types of edible insects have been consumed for hundreds of years in native communities in Asia, South America, Africa, Oceania, and Europe.[3] Nearly 2 billion people have historically used insects as a food source.[2] Recently, consuming insects has gained popularity around the world as a result of an increasing human population and environmental degradation, as well as a serious challenge in delivering enough animal-based proteins.[1] Therefore, insect farming,

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which could produce edible insects that can substitute for animal products, is currently a growing business.[4] There are various advantages to insect farming, including small-scale production, low investment, no sophisticated technology requirements, a small production area, rapid breeding cycles, and few barriers to entry.[4] Besides, insect farming is environmentally friendly and does not disrupt the balance of nature.[5] In comparison to animal farming, insect farming is more environmentally friendly because it uses less land and emits far fewer greenhouse gas emissions.[5]

Crickets are one of the insects that have received attention since crickets achieve or even exceed the recommended levels of the required essential amino acids for adults.[2] In some countries, cricket has been consumed as a fried insect and may be processed into several different forms, such as pastes, powders, and meals, which extends their shelf life and makes it simple to replace them in baking and cooking without significantly altering a dish's flavor, texture, or appearance.[2] Crickets are known to be a rich source of protein that also includes fat, which is removed from the powder in certain cases before it is processed into a diet, such as cookies, etc. and is therefore thought of as a by-product of the dietary process. Cricket has been found to contain ~60–70% w/w of protein, with fat content ranging from 10% to 23% w/w depending on the species.[6–8] The fat from insect has been used as an energy source in animal nutrition since it has been found to benefit the health of mice and pigs.[2,9] However, the applications of cricket oils in the pharmaceutical or cosmeceutical areas have not been reported before. Recently, a variety of insects, including *Hermetia illucens, Locusta migratoria, Acheta domesticus, Tenebrio molitor*, etc., have been reported for being used as bioactive ingredients in personal care and cosmetic products.[10–13] It was hence of interest to investigate the potential of using cricket oil in such pharmaceutical or cosmeceutical applications.

Thailand now has some of the most advanced cricket cultivation practices in the entire world. The number of cricket farms in Thailand is over 20,000, which provide the rural farmers with a living.[14] There are various species of crickets commercially farmed in Thailand. However, cricket farmers prefer rearing two species, including field crickets (*Gryllus bimaculatus* De Geer) and house cricket (*Acheta domesticus*).[14] Among the possibilities of raising several insect species as livestock, *G. bimaculatus* studied were the most promising species.[15] In addition, *G. bimaculatus* generally received a higher price compared to the others.[15] *G. bimaculatus* has been reported to contain 23.4 ± 0.1% w/w of lipid, which is much higher than that of *A. domesticus* (10.4 ± 0.1% w/w).[8] Therefore, *G. bimaculatus* could be used as a good source of lipid.

Nanoemulsion, primarily composed of lipids and water emulsified by emulsifiers, has become an attractive formulation with significant benefits in the cosmetics industry. Currently, nanoemulsions are increasingly being used to encapsulate lipophilic bioactive components in personal care, cosmetic, and pharmaceutical applications. The droplet diameters of nanoemulsions ranged from 50 to 1000 nm, making them resistant to sedimentation and creaming.[16] A nanoemulsion can be classified into two types based on droplet size: transparent or translucent (50–200 nm) and milky (up to 500 nm).[17] It was different from the microemulsion that requires the additional co-surfactant for reducing the interfacial tension between the lipid and water phases, resulting in the microemulsion to be a tiny internal droplet size below 100 nm and being hence transparent.[18] However, a large amount of surfactant is required for the microemulsion, whereas a lower amount of surfactant was successfully used to emulsify the nanoemulsion.[19] The present study aimed to extract the oil from *G. bimaculatus*, investigate the potential for topical applications with cosmeceutical properties, and develop nanoemulsions in order to enhance the cosmeceutical properties of *G. bimaculatus* oil.

Materials and Methods

G. bimaculatus Oil Material

Frozen adult *G. bimaculatus* (45 days old) was purchased from a cricket farm in Chiang Dao, Chiang Mai, Thailand, during the summer season (April 2022). The frozen *G. bimaculatus* was then dried using a freeze-dryer (Alpha 1–4 LDplus, Marin Christ, Osterode, Germany). The dried *G. bimaculatus* was extracted for oil by cold pressing using an oil extraction machine (FEA-100SS-M-H-TC, Energy Friend Ltd., Part., Chiang Mai, Thailand) with 220 volts, 1/2 horsepower, and 1450 rpm at an ambient temperature. The *G. bimaculatus* oil was kept in a well-closed container for further study.

Chemical Materials

2,2'-Azino-bis(3-ethylbenzthia zoline-6-sulphonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 6-hydroxy-2,5,7,8-tetramethyl

chroman-2-carboxylic acid (Trolox), epigallocatechin-3-gallate (EGCG), hyaluronic acid, ascorbic acid, kojic acid, linoleic acid, bovine serum albumin (BSA), L-tyrosine, L-dihydoxyphenylalanine (L-DOPA), Tris base, tyrosinase from mushroom (EC 1.14.18.1), matrix metalloproteinase-1 (MMP-1) from *Clostridium histolyticum* (ChC–EC.3.4.23.3), elastase from porcine pancreas (EC. 3.4.21.36), hyaluronidase from bovine testes (EC 3.2.1.35), 2-furanacryloyl-Leu-Gly-Pro-Ala (FALGPA), N-succinyl-ala-ala-ala-p-nitroanilide (AAAPVN), polysorbate 80 (Tween® 80), and sorbitan oleate (Span® 80) were analytical-grade chemicals purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin, and streptomycin were purchased from GIBCO Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Biochrom AG (Berlin, Germany). Potassium persulfate ($K_2S_2O_8$), ferric chloride ($FeCl_3$), ferrous chloride ($FeCl_2$), ammonium thiocyanate (NH_4SCN), sodium acetate, acetic acid, sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), and sodium lauryl sulphate (SLS) were analytical-grade chemicals purchased from Fisher Chemicals (Loughborough, UK). Methanol, ethanol, ethyl acetate, hexane, and dimethyl sulfoxide (DMSO) were analytical-grade solvents purchased from RCI Labscan Co., Ltd. (Bangkok, Thailand).

Determination of Fatty Acid Compositions *G. bimaculatus* Oil

G. bimaculatus oil was investigated for the fatty acid compositions using a fatty acid methyl ester/gas chromatographic-mass spectrometric (FAME/GC/MS) method. Prior to the determination, FAME was generated through the acid catalyzed transesterification of lipids with methanol. In brief, the *G. bimaculatus* oil was saponified with a 0.5 M NaOH methanolic solution at 100 °C for 15 min and derivatized for 1 min with boron trifluoride (BF_3) once it cooled down to the ambient temperature. Hexane was then added, along with the saturated NaCl aqueous solution, and the FAME that partitioned into the hexane phase was collected for further injection into the GC/MS. The GC/MS analyses were performed under the Halal GMP/HACCP and Halal-QHS/ISO 22000 by the Research and Service Laboratory, The Halal Science Center, Chulalongkorn University, Bangkok, Thailand.[20]

Antioxidant Activity Determination of *G. bimaculatus* Oil

2,2'-Azino-bis(3-ethylbenzthia zoline-6-sulphonic acid (ABTS) Assay

G. bimaculatus oil was investigated for the $ABTS^{\cdot+}$ scavenging activity by using ABTS assay.[21] In brief, ABTS solution (7 μM) and $K_2S_2O_8$ solution (2.45 mM) were combined and kept in the dark overnight to generate the stock $ABTS^{\cdot+}$ solution, which must be diluted 20 times with ethanol before the experiments. Different concentrations of *G. bimaculatus* oil were dissolved in ethyl acetate before being combined with $ABTS^{\cdot+}$ solution at a volume ratio of 1:9 to create a final volume of 200 μL. After the incubation at an ambient temperature for 5 min, the absorbance was measured at 750 nm with a DTX 880 Multimode Detector (Beckman Coulter GmbH, Austria). The experiment was repeated three times. The following equation was used to calculate the percentage of $ABTS^{\cdot+}$ scavenging activity: $ABTS^{\cdot+}$ scavenging activity (%) = [1 - (*a* / *b*)] x 100, where *a* is the absorbance of $ABTS^{\cdot+}$ solution with sample and *b* is the absorbance of $ABTS^{\cdot+}$ solution without sample. The IC_{50} value was calculated using GraphPad Prism program version 2.01 (GraphPad Software Inc., La Jolla, CA, USA). Ascorbic acid was used as a positive control.

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) Assay

G. bimaculatus oil was investigated for the $DPPH^{\cdot}$ scavenging activity by using DPPH assay.[22] In brief, different concentrations of *G. bimaculatus* oil were dissolved in ethyl acetate before being combined with $DPPH^{\cdot}$ solution (167 μM) at a volume ratio of 1:9 to create a final volume of 200 μL. After the incubation at an ambient temperature for 30 min, the absorbance was measured at 520 nm with a DTX 880 Multimode Detector (Beckman Coulter GmbH, Austria). The experiment was repeated three times. The following equation was used to calculate the percentage of $DPPH^{\cdot}$ scavenging activity: $DPPH^{\cdot}$ scavenging activity (%) = [1 - (*a* / *b*)] x 100, where *a* is the absorbance of $DPPH^{\cdot}$ solution with sample and *b* is the absorbance of $DPPH^{\cdot}$ solution without sample. The IC_{50} value was calculated using GraphPad Prism program version 2.01 (GraphPad Software Inc., La Jolla, CA, USA). Ascorbic acid was used as a positive control.

Ferric Reducing Antioxidant Power (FRAP) Assay

G. bimaculatus oil was investigated for the ferric reducing antioxidant power by using FRAP assay.[23] In brief, FRAP reagent was freshly created by combining 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$, and 300 mM acetate buffer pH 3.6 at a volume ratio of 1:1:10. Different concentrations of *G. bimaculatus* oil were dissolved in ethyl acetate before being combined with FRAP solution at a volume ratio of 1:9 to create a final volume of 200 µL. After the incubation at an ambient temperature for 5 min, the absorbance was measured at 595 nm with a DTX 880 Multimode Detector (Beckman Coulter GmbH, Austria). The experiment was repeated three times. The following equation was used to calculate the percentage of ferric reducing ability: ferric reducing ability (%) = [1 - (*a* / *b*)] x 100, where *a* is the absorbance of FRAP solution with sample and *b* is the absorbance of FRAP solution without sample. The IC_{50} value was calculated using GraphPad Prism program version 2.01 (Graphpad Software Inc., La Jolla, CA, USA). Ascorbic acid was used as a positive control.

Ferric Thiocyanate (FTC) Assay

G. bimaculatus oil was investigated for the lipid peroxidation inhibition by using FTC assay.[24] In brief, different concentrations of *G. bimaculatus* oil were dissolved in ethyl acetate before being combined with 50% v/v linoleic acid in DMSO, 10% w/v ammonium thiocyanate (NH_4SCN) aqueous solution, and 2 mM ferrous chloride ($FeCl_2$) aqueous solution at a volume ratio of 1:1:1:1 to create a final volume of 200 µL. After the incubation at 37 ± 2 °C for 5 min, the absorbance was measured at 490 nm with a DTX 880 Multimode Detector (Beckman Coulter GmbH, Austria). The experiment was repeated three times. The following equation was used to calculate the percentage of lipid peroxidation inhibition: lipid peroxidation inhibition (%) = [1 - (*a* / *b*)] x 100, where *a* is the absorbance of the mixture with sample and *b* is the absorbance of the mixture without sample. The IC_{50} value was calculated using GraphPad Prism program version 2.01 (GraphPad Software Inc., La Jolla, CA, USA). Trolox was used as a positive control.

Anti-Tyrosinase Activity Determination of *G. bimaculatus* Oil

G. bimaculatus oil was investigated for its inhibitory activities against the tyrosinase enzyme on the degradation of two key substrates of the melanogenic pathway, L-tyrosine and L-DOPA, using an in vitro enzyme-substrate assay.[25] In brief, different concentrations of *G. bimaculatus* oil were dissolved in ethyl acetate before being combined with the tyrosinase enzyme at a volume ratio of 1:3 to create a final volume of 40 µL. After the incubation at an ambient temperature for 10 min, 100 µL of 2.5 mM L-tyrosine or L-DOPA was added and incubated for another 30 min. The absorbance was measured at 492 nm with a DTX 880 Multimode Detector (Beckman Coulter GmbH, Austria). The experiment was repeated three times. The following equation was used to calculate the percentage of tyrosinase inhibition: tyrosinase inhibition (%) = [1 - (*a* / *b*)] x 100, where *a* is the absorbance of the mixture with sample and *b* is the absorbance of the mixture without sample. The IC_{50} value was calculated using GraphPad Prism program version 2.01 (GraphPad Software Inc., La Jolla, CA, USA). Kojic acid was used as a positive control.

Anti-Skin Ageing Activity Determination of *G. bimaculatus* Oil

Determination of Collagenase Inhibitory Activity

G. bimaculatus oil was investigated for its inhibitory activities against the collagenase enzyme (*C. histolyticum* matrix metalloproteinase-1, MMP-1) on the degradation of FALGPA.[26] In brief, different concentrations of *G. bimaculatus* oil were dissolved in ethyl acetate before being combined with the 0.1 U/mL collagenase solution at a volume ratio of 1:1 to create a final volume of 40 µL. After the incubation at an ambient temperature for 15 min, 120 µL of FALGPA in 50 mM tricine buffer was added. Immediately, the absorbance was kinetically measured at 340 nm with a DTX 880 Multimode Detector (Beckman Coulter GmbH, Austria) for 10 min. The experiment was repeated three times. The following equation was used to calculate the percentage of collagenase inhibition: collagenase inhibition (%) = [1 - (*a* / *b*)] x 100, where *a* is the absorbance of the mixture with sample and *b* is the absorbance of the mixture without sample. The IC_{50} value was calculated using GraphPad Prism program version 2.01 (GraphPad Software Inc., La Jolla, CA, USA). EGCG was used as a positive control.

Determination of Elastase Inhibitory Activity

G. bimaculatus oil was investigated for its inhibitory activities against the elastase enzyme on the degradation of AAAPVN.[26] In brief, different concentrations of *G. bimaculatus* oil were dissolved in ethyl acetate before being combined with the 0.03 U/mL elastase solution at a volume ratio of 1:4 to create a final volume of 50 µL. After the incubation at an ambient temperature for 15 min, 150 µL of AAAPVN in 200 mM Tris-HCL buffer pH 8.0 was added. Immediately, the absorbance was kinetically measured at 410 nm with a DTX 880 Multimode Detector (Beckman Coulter GmbH, Austria) for 10 min. The experiment was repeated three times. The following equation was used to calculate the percentage of elastase inhibition: elastase inhibition (%) = [1 - (*a* / *b*)] x 100, where *a* is the absorbance of the mixture with sample and *b* is the absorbance of the mixture without sample. The IC_{50} value was calculated using GraphPad Prism program version 2.01 (GraphPad Software Inc., La Jolla, CA, USA). EGCG was used as a positive control.

Determination of Hyaluronidase Inhibitory Activity

G. bimaculatus oil was investigated for its inhibitory activities against the hyaluronidase enzyme on the degradation of hyaluronic acid.[27] In brief, different concentrations of *G. bimaculatus* oil were dissolved in ethyl acetate before being combined with the 15 unit/mL hyaluronidase solution at a volume ratio of 1:5 to create a final volume of 120 µL. After the incubation at 37 °C for 10 min, 0.03% w/v hyaluronic acid in phosphate buffer pH 5.35 was added and incubated again at 37 °C, for 45 min. The absorbance was measured at 600 nm with a DTX 880 Multimode Detector (Beckman Coulter GmbH, Austria) after the addition of acid bovine serum albumin, which was prepared by mixing sodium acetate with acetic acid and bovine serum albumin. The experiment was repeated three times. The following equation was used to calculate the percentage of hyaluronidase inhibition: hyaluronidase inhibition (%) = [1 - (*a* / *b*)] x 100, where *a* is the absorbance of the mixture with sample and *b* is the absorbance of the mixture without sample. The IC_{50} value was calculated using GraphPad Prism program version 2.01 (GraphPad Software Inc., La Jolla, CA, USA). Oleanolic acid was used as a positive control.

Cytotoxicity Effect Determination of *G. bimaculatus* Oil

Cells Culture

The immortalized human epidermal keratinocyte cell line (HaCaT cells, American Type Culture Collection, VA, USA) and human foreskin fibroblasts (Hs68 cells, Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan), which were stored frozen at the Faculty of Associated Medical Sciences, Chiang Mai University, Thailand, were thawed into liquid and continually kept cold in the ice bath. The thawed cells were resuspended in the DMEM cell-culture medium supplemented with 10% v/v fetal calf serum, 1 mM L-glutamine, 100 unit/mL penicillin, and 100 µg/mL streptomycin. Prior to the cell culture, the cryoprotectant was removed after centrifugation at 1000 rpm for 3 min. The cells were resuspended in a fresh medium and incubated at 37 °C with 5% CO_2 and subsequently split with a mixture of 0.02% w/v trypsin and 0.02% w/v EDTA until confluence was attained.

Cytotoxicity Test by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

The effects of *G. bimaculatus* oil on the viability of HaCaT and Hs68 cells were examined using the MTT assay in order to establish the range of cytotoxic doses. HaCaT cells were seeded at a density of 3×10^5 cells per well, whereas Hs68 cells were seeded at a density of 3.5×10^5 cells per well and incubated at 37 °C with 5% CO_2 for 24 h. The cells were then treated with *G. bimaculatus* oil at various concentrations ranging from 0 to 100 µg/mL and further incubated at 37 °C with 5% CO_2 for 48 h. Subsequently, 100 µL of the culture media was discarded and 15 µL of a 5 mg/mL MTT dye solution was added and further maintained for 4 h. After the supernatants were discarded, 200 µL of DMSO was added to solubilize the formazan complex and measured for absorbance at 578 nm and 630 nm using a microplate reader (Bio Tek Instruments, USA).

Irritation Test of *G. bimaculatus* Oil by Hen's Egg Chorio-Allantoic Membrane (HET-CAM) Test

The ability of *G. bimaculatus* oil to irritate was investigated in fertilized hen's eggs by using the HET-CAM test.[28] The hen's eggs, which have been fertilized for 7–10 days, were incubated at 37.5 ± 0.5 °C and 62.5 ± 7.5% relative humidity in an

automated rotating egg incubator (Nanchang Howard Technology Co., Ltd., Jiangxi, China). The egg's shell above the air cell was opened using a spinning cutting blade attached to a dental micromotor (Marathon-3 Champion, Saeyang Microtech, Daegu, South Korea). Normal saline solution was then dropped onto the inner membrane, which was directly in contact with the CAM, making the CAM moist and easily removed. After carefully removing the CAM, 30 µL of the sample was applied. The irritation signs, including hemorrhage, vascular lysis, and coagulation, were thoroughly observed under the stereo microscope (Olympus, Tokyo, Japan) for 300 s. Each irritation sign's initial appearance was noted, and the irritation score (*IS*) was then computed using the following equation: $IS = [(301 - h) \times 5]/300 + [(301 - l) \times 7]/300 + [(301 - c) \times 9]/300$, where *h* is the second of first appeared hemorrhage, *l* is the second of first appeared vascular lysis, and *c* is the second of first appeared coagulation. The IS could be divided into four categories: non-irritation (0.0–0.9), modest irritation (1.0–4.9), moderate irritation (5.0–8.9), and severe irritation (9.0–21.0).[29] The experiment was repeated three times. SLS aqueous solution (1% w/v) and normal saline solution were used as a positive and a negative control, respectively.

Determination Required Hydrophilic–Lipophilic Balance (RHLB) of *G. bimaculatus* Oil

The RHLB of *G. bimaculatus* oil was determined following the method previously described by Chaiyana et al,[30] using two emulsifiers, polysorbate 80 (HLB = 15) and sorbitan oleate (HLB = 4.3), in different proportions to obtain the HLB in the range of 8 to 12. Subsequently, a mixture of polysorbate 80 and sorbitan oleate was mixed with *G. bimaculatus* oil and DI water and thoroughly mixed using a vortex mixer Genie 2 (Scientific Industries, NY, USA) for 20 min. After that, each formulation was set aside for 24 h and the homogeneity or phase separation was observed. The RHLB of *G. bimaculatus* oil was defined from the mixture showing the least phase separation.

Development of Nanoemulsions from *G. bimaculatus* Oil

Nanoemulsion Preparation Method

G. bimaculatus oil was used to develop a nanoemulsion using the combination of polysorbate 80 and sorbitan oleate that yielded the equivalent HLB to RHLB of the oil. The composition of each nanoemulsion is shown in Table 1. *G. bimaculatus* oil and sorbitan oleate were combined to yield an oil phase. On the other hand, DI water and polysorbate 80 were combined to yield a water phase. The primary emulsion was then generated by adding the oil phase into the water phase and homogenizing at a high speed of 12,000 rpm for 5 min using a high-speed homogenizer (IKA® T25 digital Ultra-Turrax, Staufen, Germany). Subsequently, the resulting primary emulsion was passed through a high-pressure homogenizer (APV 1000, Wilmington, MA, USA) set at 400 bars for 7 cycles. The nanoemulsion was kept in a well-closed container until further use.

Nanoemulsion Characterization

The nanoemulsions of *G. bimaculatus* oil were characterized for the internal droplet size, size distribution (polydispersity, PDI), and zeta potential, which were analyzed using photon correlation spectroscopy (Zetasizer, version 5.00, Malvern Instruments Ltd., Malvern, UK). The results were presented as the mean and standard deviation of at least 10 measurements on each sample. The viscosity of nanoemulsions was evaluated using a rheometer equipped with an R/S spindle CC48 (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) at 25 °C. Hitachi HT-7700

Table 1 The Composition of Each Nanoemulsions

Ingredients	Concentration (% w/w)		
	F1	F2	F3
G. bimaculatus oil	1	1	1
Sorbitan oleate	1.12	1.40	1.68
Polysorbate 80	0.88	1.10	1.32
DI water q.s.	100	100	100

Abbreviations: F1, nanoemulsion formulation 1; F2, nanoemulsion formulation 2; F3, nanoemulsion formulation 3.

transmission electron microscope (Hitachinaka, Ibaraki, Japan) was used to investigate the morphology of the nanoemulsion. For sample preparation, a drop of the nanoemulsion was carefully deposited onto a copper grid coated with carbon. A negative stain (20 µL of uranyl acetate 2% w/v solution) was introduced into the mixture and subsequently allowed to incubate for 20 min at room temperature to enhance the contrast of image. Thereafter, any excess liquid was meticulously removed with a piece of filter paper, and the samples were allowed to rest in a desiccator overnight to achieve complete removal of the solvent. Finally, images were captured with the TEM operating at 40k and 50k magnification.

Stability Test of Nanoemulsion

Each nanoemulsion underwent 8 heating-cooling cycles, lasting 24 h each at 4 °C and 45 °C, before being assessed for external appearance, particle size, PDI, and zeta potential, as previously mentioned.

Cosmeceutical Properties of Nanoemulsions from *G. bimaculatus* Oil

The nanoemulsion of *G. bimaculatus* oil was investigated for cosmeceutical properties in comparison to the native *G. bimaculatus* oil at the same concentration of 1% w/w in terms of inhibitory activities against lipid peroxidation, tyrosinase, collagenase, elastase, and hyaluronidase using the method described above.

Statistical Analysis

All data were presented as a mean ± standard deviation (S.D.). Statistical significance was assessed by the one-way analysis of variance (ANOVA) followed by post hoc tests or a paired-samples *t*-test (in the case of two matched groups of data) using the GraphPad Prism program version 2.01 (GraphPad Software Inc., La Jolla, CA, USA). The probability values of * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered significant.

Results and Discussion

Fatty Acid Compositions of *G. bimaculatus* Oil

The oil extracted from *G. bimaculatus* (Figure 1A) is a dark brown liquid (Figure 1B). It has a unique smell that resembles food. One hundred grams of frozen *G. bimaculatus* yielded 34.0 g of dried *G. bimaculatus*, which yielded 7.5 g of *G. bimaculatus* oil. Therefore, the oil obtained from dried *G. bimaculatus* was accounted for as 22.1% w/w of its dried mass. The results were in line with the previous study, which reported that *G. bimaculatus* contained 23.4 ± 0.1% w/w of lipid.[9] The fatty acid composition of *G. bimaculatus* is shown in Table 2. The amount of saturated fatty acids (SFAs) was comparable to that of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), which are 34.36 ± 0.01%, 32.98 ± 0.01%, and 32.66 ± 0.01%, respectively. It was noted that the amount of unsaturated fatty acids (USFAs) was significantly greater than that of SFAs. The results were in line with the previous study by Kim et al, which reported that the content of USFAs (60.74 ± 0.4 to 63.55 ± 0.56%

Figure 1 Dried *G. bimaculatus* (**A**) and the oil extracted from *G. bimaculatus* (**B**).

Table 2 The Fatty Acid Composition of *G. bimaculatus* Oil

Fatty Acids		Amount (%)
Saturated fatty acids		34.36 ± 0.01
C12:0	Lauric acid	0.09 ± 0.00
C14:0	Myristic acid	0.63 ± 0.01
C15:0	Pentadecanoic acid	0.07 ± 0.00
C16:0	Palmitic acid	24.80 ± 0.01
C17:0	Hepadecanoic acid	0.16 ± 0.00
C18:0	Stearic acid	7.61 ± 0.00
C20:0	Arachidic acid	0.59 ± 0.00
C21:0	Heneicosanoic acid	0.08 ± 0.01
C22:0	Behenic acid	0.25 ± 0.00
C23:0	Tricosanoic acid	0.08 ± 0.00
C24:0	Lignoceric acid	0.03 ± 0.01
Unsaturated fatty acids		65.64 ± 0.01
Monounsaturated fatty acids		32.98 ± 0.01
C16:1n7	Palmitoleic acid	1.16 ± 0.00
C17:1n10	cis-10-Heptadecenoic acid	0.08 ± 0.01
C18:1n9c	cis-9-Oleic acid	30.44 ± 0.01
C18:1n9t	Tans-9-Elaidic acid	0.27 ± 0.00
C20:1n11	cis-11-Eucosenoic acid	0.27 ± 0.00
C22:1n9	Erucic acid	0.54 ± 0.00
C24:1n9	Nervonic acid	0.23 ± 0.01
Polyunsaturated fatty acids		32.66 ± 0.01
C18:2n6t	Linoleic acid	31.08 ± 0.00
C18:3n3	Alpha-Linolinic acid	1.09 ± 0.01
C18:3n6	Gamma-Linolinic acid	0.16 ± 0.00
C20:2	Eicosadienoic acid	0.07 ± 0.00
C20:3n3	Eicosatrienoic acid	0.18 ± 0.00
C20:4n6	Arachidonic acid	0.01 ± 0.00
C20:5n3	Docosapentaenoic acid	0.015 ± 0.01
C22:2	Docosadienoic acid	0.03 ± 0.00

w/w) was double that of SFAs (29.19 ± 0.79 to 31.88 ± 0.56% w/w) in *G. bimaculatus* powder from various processing conditions, including lyophilization process, hot-air process at 90 °C for 7 h, roasting process at 160 °C for 40 min, and frying process at 180 °C for 30 sec.[31] However, the results differed from the previous study by Udomsil et al, which reported that the ratio of SFAs: MUFAs: PUFAs was 12.76: 9.85: 1.80 in *G. bimaculatus* powder from an air-dried process at 60 °C for 48 h.[8]

When examining fatty acids one at a time, linoleic acid (31.08 ± 0.00%) was found to be the most prominent fatty acid in *G. bimaculatus* oil, followed by oleic acid (30.44 ± 0.01%), palmitic acid (24.80 ± 0.01%), stearic acid (7.61 ± 0.00%), etc. All the findings were in excellent accord with the earlier research by Kim et al.[31] Interestingly, the fatty acid components of *G. bimaculatus* oil were in line with many cosmetic oils of plant origin. Bialek et al examined the fatty acid components of 17 cosmetic oils and found that palmitic acid and stearic acid were the main SFAs, with amounts ranging from 3.2% to 33.5%.[32] Furthermore, the main MUFA detected in all cosmetic oils examined was oleic acid, while the main PUFAs were linoleic acid and alpha-linolenic acid.[32]

The primary constituent of *G. bimaculatus* oil, linoleic acid, makes it suited for cosmetic purposes since linoleic acid has been reported to be a constituent of ceramides, the skin fat that decreases with age and results in skin roughness and sensitivity.[32] Fatty acids, particularly linoleic acid, therefore played a crucial role and were essential for maintaining the skin's normal structure and functions. Aside from being a constituent of skin fat, linoleic acid showed a beneficial effect on the skin by attenuating cutaneous inflammation via competition with the inflammatory arachidonic acid and proinflammatory eicosanoid inhibition.[33] Furthermore, linoleic acid regulated the synthesis and activity of cytokines to promote wound healing.[33]

Antioxidant Activities of *G. bimaculatus* Oil

Oxidative stress, defined as an imbalance between reactive oxygen species and antioxidant defense and repair mechanisms, has been linked to the emergence of generative diseases and the oxidative damages that can exacerbate skin pigmentation and ageing, inducing changes in skin complexion homogeneity, wrinkling, sagging, dryness, and roughness, as well as skin ageing and wrinkles.[34] Antioxidants therefore considered to be a promising strategy to counteract skin ageing. Four distinct assays were used to investigate the antioxidant abilities of *G. bimaculatus* oil in the present study, as there are a variety of mechanisms to decrease or stop the oxidation process. Free radical generation is directly related to oxidation, so scavenging the free radicals might be a tactical way to stop the oxidation process.[35] On the other hand, FRAP measures the capacity of an antioxidant to reduce an oxidant via the transfer of a single electron, which is based primarily on the deprotonation and ionization potential of the reactive functional group.[36] Therefore, individuals with reducing abilities might slow down or terminate the oxidative-chain reaction. On the other aspect, oxidation on lipids produces lipid oxidation products that are carcinogenic and cause photocarcinogenesis.[37] Antioxidants should thus be advantageous for preserving health and lowering the risk of disease by inhibiting lipid peroxidation.[38]

The antioxidant activities of *G. bimaculatus* oil, as shown in Figure 2, were dose-dependent in terms of free radical scavenging, ferric reducing, and lipid peroxidation inhibitory abilities. It was noted that *G. bimaculatus* oil has the most potential to inhibit the oxidation process via its reducing ability, since the IC_{50} value for ferric reducing power was the

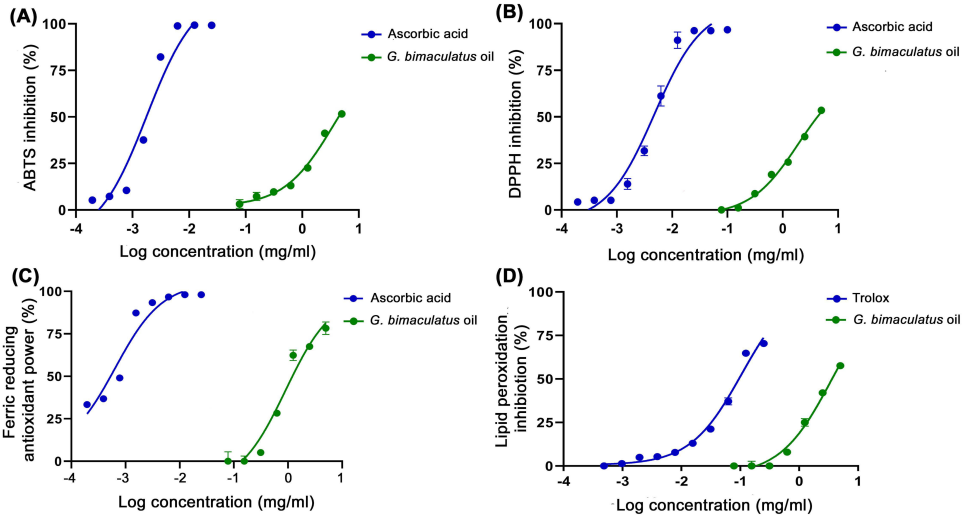

Figure 2 Dose response curves on 2,2'-azino-bis(3-ethylbenzthia zoline-6-sulphonic acid (ABTS) inhibition (**A**), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) inhibition (**B**), ferric reducing antioxidant power (**C**), and lipid peroxidation inhibition (**D**) of *G. bimaculatus* oil, ascorbic acid, and Trolox.

Table 3 Biological Activities Related to Cosmeceutical Applications of *G. bimaculatus* Oil

Biological Activities	IC_{50} (μg/mL)		Type of Positive Control
	***G. bimaculatus* Oil**	**Positive Control**	
Antioxidant activities			
ABTS inhibition	3540.7 ± 652.9	1.4 ± 0.0	Ascorbic acid
DPPH inhibition	1333.0 ± 68.4	4.8 ± 0.4	Ascorbic acid
Ferric reducing power	643.2 ± 2.9	0.6 ± 0.0	Ascorbic acid
Lipid peroxidation	2067.7 ± 392.7	107.0 ± 6.8	Trolox
Anti-tyrosinase activities			
Substrate: L-Tyrosine	172.2 ± 43.9	10.8 ± 0.6	Kojic acid
Substrate: L-DOPA	837.6 ± 194.5	4.0 ± 0.1	Kojic acid
Anti-skin ageing activities			
Collagenase inhibition	63.9 ± 6.3	18.1 ± 1.5	EGCG
Elastase inhibition	178.6 ± 62.0	31.8 ± 1.3	EGCG
Hyaluronidase inhibition	813.4 ± 245.6	3.1 ± 0.4	Oleanolic acid

Abbreviations: IC_{50}, half-maximal inhibitory concentration; ABTS, 2,2'-azino-bis(3-ethylbenzthia zoline-6-sulphonic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; EGCG, Epigallocatechin gallate.

lowest (643.2 ± 2.9 mg/mL) when compared to other mechanisms (Table 3). However, its antioxidant activities were much lower than those of the positive controls, requiring a higher concentration to achieve the same effects as ascorbic acid and Trolox. Additionally, the results from Figure 2 showed that *G. bimaculatus* oil, ascorbic acid, and Trolox exhibited a dose-dependent relationship, indicating that higher concentrations would result in higher antioxidant effects. Besides, IC_{50} value could be used as an indicator for the concentration used in the formulation.

Anti-Tyrosinase Activities of *G. bimaculatus* Oil

Tyrosinase, a crucial enzyme in the production of melanin, plays a key role in the first steps of the pathway, involving the hydroxylation of L-tyrosine via its monophenolase activity and the oxidation of L-DOPA to produce *o*-dopaquinone, which leads to the skin's darkness.[39] The compounds with anti-tyrosinase activity are promising cosmetic and pharmaceutical ingredients to prevent overproduction of melanin in epidermal layers or as whitening agents.[39] The anti-tyrosinase activities of *G. bimaculatus* oil on the hydroxylation of L-tyrosine and the oxidation of L-DOPA are shown in Figure 3. The inhibitions of tyrosinase were dose dependent. Comparing to kojic acid, an anti-melanogenic agent, *G. bimaculatus* oil possessed lower potential. However, kojic acid, which is the most-commonly used in the cosmetics industry, has been linked to probable mutagenesis effects as well as negative side effects such skin irritation and contact

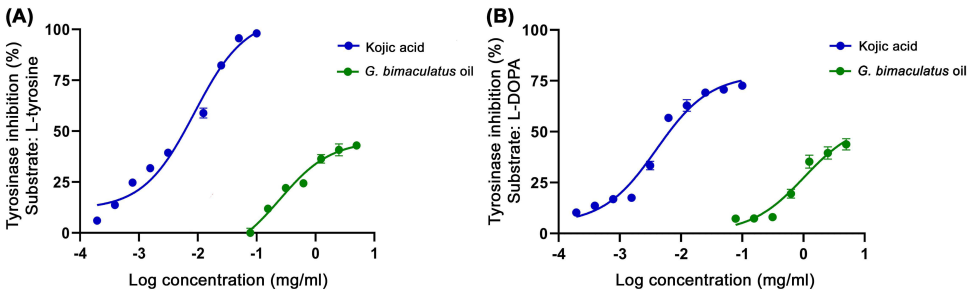

Figure 3 Dose response curves on tyrosinase inhibition when the substrate was L-tyrosine (**A**) and L-DOPA (**B**) of *G. bimaculatus* oil and kojic acid.

dermatitis.[40] Therefore, finding out the natural compounds that have anti-tyrosinase activities and are safe is of interest. Although *G. bimaculatus* oil did not possess a potent anti-tyrosinase, it has a potential to inhibit the enzyme activities. Since the IC_{50} of L-tyrosine hydroxylation against tyrosinase was 172.2 ± 43.9 µg/mL (Table 3), or roughly 0.02% w/w, a much higher concentration, resulting in higher efficacy, could be used in the cosmetic formulation. Or even the IC_{50} of L-DOPA oxidation, which was 837.6 ± 194.5 µg/mL (Table 3), was roughly accounted for at 0.08% w/w. Therefore, *G. bimaculatus* oil has a promising whitening effect via its anti-tyrosinase activity, though high concentrations need to be used in the formulations.

Anti-Skin Ageing Activities of *G. bimaculatus* Oil

Skin ageing can be caused by both intrinsic and extrinsic factors. Ultraviolet radiation, in particular, is one extrinsic factor that contributes to premature wrinkles.[41] The disordered and clumped collagen found in photo-aged skin is most likely the result of long-term increases in the levels of MMPs, notably MMP-1.[42] Furthermore, the deposition of elastin is thought to be pathognomonic of photo-aged skin because alterations in elastic fibers are so intimately connected to photo-aged skin.[42] Hyaluronic acid, one of the most common glycosaminoglycans, gives normal skin a plump, smooth, and hydrated appearance and is thought to contribute to the skin's youth. It is constant in the dermis but declines almost completely with ageing in the epidermis.[42] Therefore, the approach to restoring the skin's youthfulness would be to reduce the rate at which collagen, elastin, and hyaluronan degrade by inhibiting their degradation enzymes. The inhibitory activities against collagenase, elastase, and hyaluronidase of *G. bimaculatus* oil are shown in Figure 4. *G. bimaculatus* oil inhibited the enzyme in a dose-dependent manner. The IC_{50} values in Table 3 characterized *G. bimaculatus* oil as an attractive anti-collagenase and anti-elastase agent. Although *G. bimaculatus* oil was not as potent as EGCG, which was used as a positive control, it showed a promising ability to inhibit collagenase and elastase at around 3.5 and 5.7 times the concentration of EGCG, respectively.

Cytotoxicity Effects of *G. bimaculatus* Oil

Skin comprises essentially two major types of cells, including keratinocytes and fibroblasts, which are embedded in the epidermis and dermis, respectively. HaCaT cells are typically utilized as an in vitro model for highly proliferative epidermis,[43] whereas Hs68 cells are commonly used normal fibroblast cells.[44] The cytotoxicity effects of *G. bimaculatus* oil on HaCaT and Hs68 cells is shown in Figure 5. The results showed the same cytotoxicity trend for *G. bimaculatus* oil and its major components, including linoleic acid, oleic acid, and palmitic acid, with no statistically significant difference ($p > 0.05$). HaCaT was more sensitive to oil and fatty acids than Hs68 since the HaCaT cell viability gradually decreased with increasing concentrations of the test compounds, whereas the Hs68 cell viability remained greater than 90% even at the high concentration of 100 µg/mL. Therefore, the findings from this study noted that the IC_{20} on Hs68 cells were greater than 100 µg/mL. On the other hand, the HaCaT cell viabilities were 78.7 ± 5.1%, 73.8 ± 5.8%, 82.2 ± 6.1%, and 75.9 ± 3.5% after being treated with *G. bimaculatus* oil, linoleic acid, oleic acid, and palmitic acid, respectively, after 48 h at a concentration of 100 µg/mL. As these values were not significantly different from 80% ($p > 0.05$), it could be

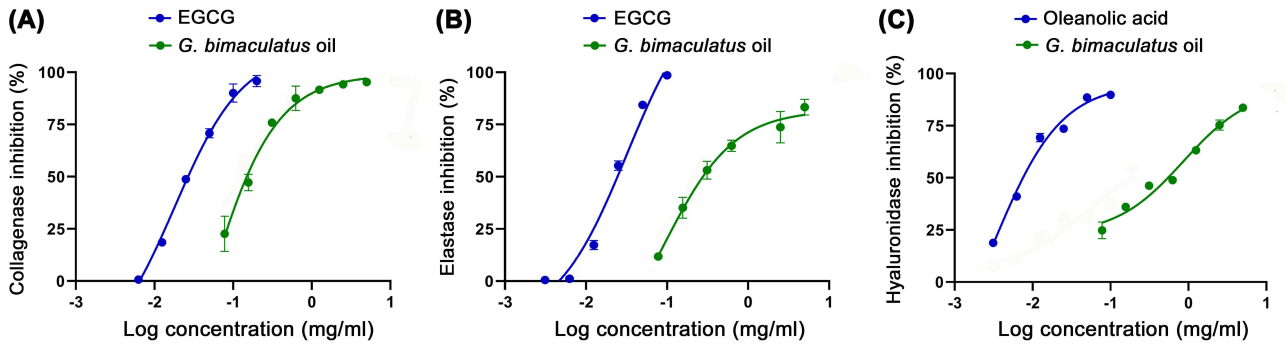

Figure 4 Dose response curves on inhibition of collagenase (**A**), elastase (**B**), and hyaluronidase (**C**) of *G. bimaculatus* oil, epigallocatechin gallate (EGCG), and oleanolic acid.

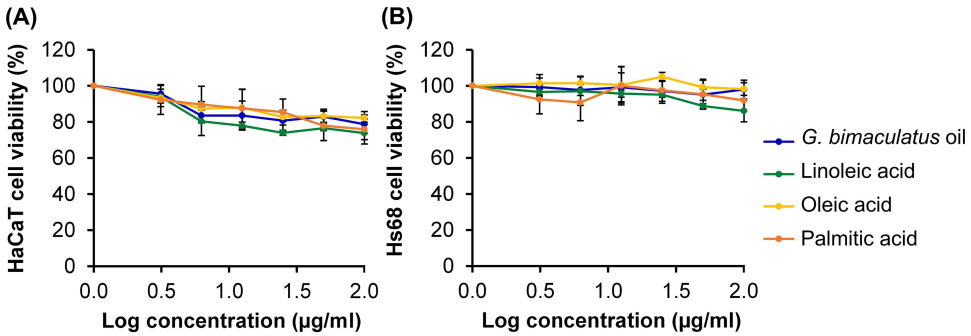

Figure 5 Cytotoxic effect of *G. bimaculatus* oil and its major fatty acid component, including linoleic acid, oleic acid, and palmitic acid, on immortalized human epidermal keratinocyte (HaCaT) cells (**A**) and human foreskin fibroblasts (Hs68) cells (**B**) after 48 h treatment. The data are demonstrated as mean ± SD (n = 3).

concluded that the IC_{20} of *G. bimaculatus* oil and each fatty acid on HaCaT cells was 100 µg/mL. Furthermore, *G. bimaculatus* oil was highlighted as being safe for the skin cells, especially the fibroblasts in the dermal layer. The suggested concentration of *G. bimaculatus* oil in the further study in cell culture was 100 µg/mL.

Irritant Effects of *G. bimaculatus* Oil

Irritant effects of *G. bimaculatus* oil on the vital vascular membrane using the HET-CAM test, an alternative to the Draize eye irritation test,[45] are shown in Figure 6. Hemorrhage, vascular lysis, and coagulation were observed in the CAM after exposure to SLS aqueous solution (1% w/v), which was used as a positive control. The calculated IS was 12.5 ± 0.3, which represents a severe irritation effect. Furthermore, a longer duration of the exposure leads to more irritating effects involving hemorrhage, vascular lysis, and coagulation. In contrast, normal saline solution (0.9% w/v NaCl solution) causes no irritation (IS = 0.0 ± 0.0) even after 60 min of exposure. The results from both positive and negative controls can be used to verify the validity of the HET-CAM method.

The IS of *G. bimaculatus* oil and its major fatty acid components, including linoleic acid, oleic acid, and palmitic acid, were also 0.0 ± 0.0, which represented no irritant effect. No signs of irritation after being exposed to *G. bimaculatus* oil were detected even after 60 min. However, hemorrhage was detected in all fatty acid and vascular lysis was detected in palmitic acid after 60 min of the exposure. Therefore, pure fatty acids tended to induce irritation, whereas *G. bimaculatus* oil, which contained a variety of fatty acids, was safe. The likely explanation could be due to the

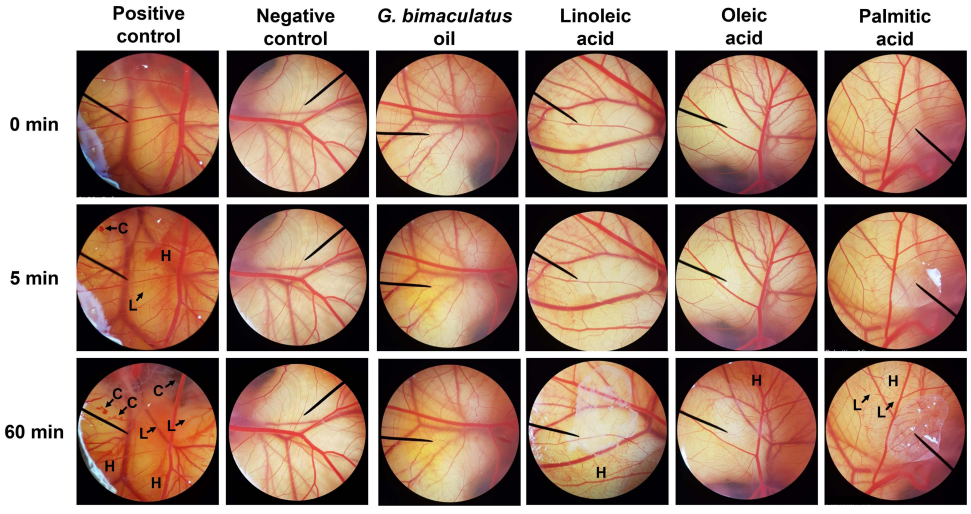

Figure 6 Effect of positive control (1% w/v sodium lauryl sulfate (SLS) aqueous solution), negative control (normal saline solution), *G. bimaculatus* oil and its major fatty acid component, including linoleic acid, oleic acid, and palmitic acid on the chorioallantoic membrane at 0, 5, and 60 min. The letter H represents hemorrhage, L represents vascular lysis, and C represents coagulation.

lower concentration of each fatty acid in *G. bimaculatus* oil, which is lower than the irritation dose. Previous studies reported that fatty acids exhibited the irritant effects in a single insult occlusive patch test in rabbits.[46,47] Acid (1987) reported that oleic acid caused a primary irritation index (PII) of 0.5 and moderate erythema after 24 h of the treatment, whereas the administration of palmitic acid yielded a PII of 0.0 and minimal erythema after 2–24 h.[48] On the other hand, Ben-Shabat et al reported that the primary PII of linoleic acid and oleic acid were about the same.[47] However, a clinical experiment using human volunteers exhibited a different irritating impact. Stillman et al investigated the effects of daily application of unsaturated C18 free-fatty acids and saturated-free fatty acids from C3 to C18 under occlusive patch testing to human skin and revealed that C8 to C12 fatty acids were the most corrosive.[48] Palmitic acid, containing 16 carbons, and oleic acid, containing 18 carbons, was not found to be irritating, but linoleic acid (C18:2) was found to produce irritation.[48] However, those findings came from an experiment done under occlusive conditions, which might have caused the irritation to be more severe. Kränke et al revealed that the compounds, which generally have a very low irritating potential, demonstrated a meaningful irritating potential when administered in an occlusive condition.[49]

RHLB of *G. bimaculatus* Oil

The RHLB of an oil or oil phase represents the ideal HLB value of the emulsifier system for developing the most stable and dispersive emulsion.[50] Since there has been no report about the RHLB of *G. bimaculatus* oil, this is the first study to reveal the ideal HLB value of the emulsifier system for the emulsion development from *G. bimaculatus* oil.

Different emulsion formulations were developed using *G. bimaculatus* oil as an oil phase and emulsified by a mixture of polysorbate 80 and sorbitan oleate with various HLB values ranging from 9 to 12. The results noted that an emulsion with an HLB value of 9 demonstrated the least amount of phase separation, as shown in Figure 7. As a result, it could be concluded that the RHLB of *G. bimaculatus* oil was 9. Since an emulsifier system with an HLB value equal to the RHLB of the *G. bimaculatus* oil was suggested for use in the emulsion formulation, the ratio of polysorbate 80 and sorbitan oleate of 0.56 to 0.44 was used in the further development of nanoemulsion.

Nanoemulsions from *G. bimaculatus* Oil

Nanoemulsions were successfully developed using 1% w/w of *G. bimaculatus* oil. The concentration of *G. bimaculatus* oil in the formulation was found to be higher than 10 times the IC_{50} value for anti-tyrosinase, anti-collagenase, anti-elastase, anti-hyaluronidase, and ferric reducing antioxidant power. These findings suggest that the nanoemulsion formulation of *G. bimaculatus* oil may be effective in providing biological effects against these enzymes and in exhibiting antioxidant

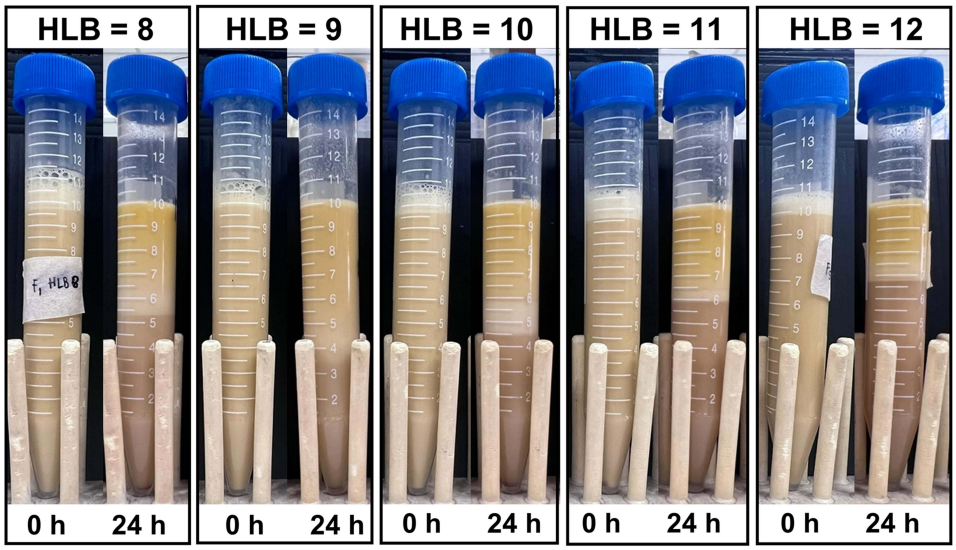

Figure 7 The external appearance of the emulsion of *G. bimaculatus* oil using different emulsifier systems has the hydrophilic–lipophilic balance (HLB) ranging from 8 to 12 after the preparation and after 24 h.

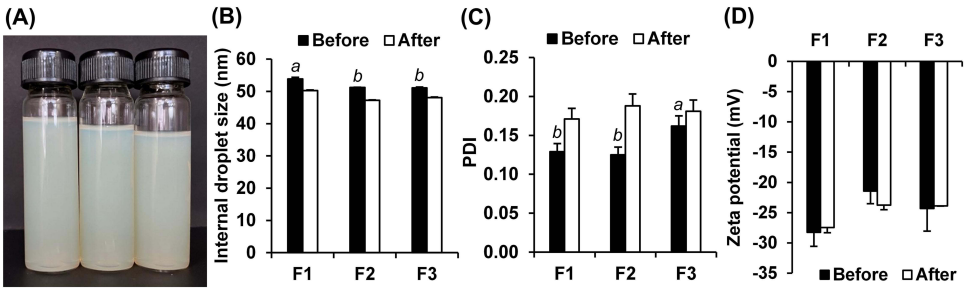

Figure 8 The external appearance of the emulsion of *G. bimaculatus* oil using the emulsifier systems with hydrophilic–lipophilic balance (HLB) of 9 (**A**), internal droplet size (**B**), polydispersity index (PDI) (**C**), and zeta potential (**D**) before and after an accelerated stability test in 8 heating-cooling cycles. The letters (a and b) indicate a statistically significant difference between nanoemulsion containing *G. bimaculatus* oil formulations F1, F2, and F3 ($P<0.05$).

activity, as determined by the ferric reducing antioxidant power assay. All nanoemulsions were translucent liquid as shown in Figure 8. All nanoemulsions exhibited Newtonian flow behavior, with the viscosity in the range of 0.089 ± 0.013 to 0.105 ± 0.003 mPas. The low viscosity would lead to ease of application on the skin. The external appearance of the emulsion shifts from a milky white color, which was due to the light scattering caused by the difference in refractive indices between the internal and external phases, to a translucent bluish color when the particle size is reduced.[51] In general, an internal droplet size greater than 1 μm resulted in an opaque emulsion.[51] Therefore, the translucent formulation could be defined as a nanoemulsion via visual inspection. Besides, the internal droplet size of the nanoemulsions from *G. bimaculatus* oil was investigated by dynamic light scattering measurements, which found that the sizes were in the nano-range of 51.09 ± 0.25 to 53.84 ± 0.55 nm. The TEM micrographs as shown in Figure 9 could be used to confirm the internal droplet size of the nanoemulsion. The results were consistent with previous research,[52] which found that colloidal systems with sizes ranging from 21.8 to 62.0 nm were translucent due to the difference in refractive indexes between the polymer particles and the continuous aqueous phase, even when the diameter of a nanoparticle is smaller than the wavelength of visible light (400–750 nm), which should be transparent and not optically detected.

Since the internal droplet size is the key factor affecting the efficacy of the system regarding the delivery of the active compound into the skin layer, different droplet sizes would result in different potencies. While the contents of nanovesicles with a diameter of 70 nm or smaller could transfer the compounds into both the dermal and epidermal layers, those with larger diameter up to 300 nm could only deliver the contents into the deeper skin layers and the vesicles having a diameter of 600 nm or larger are often unable to deliver their contents into the skin's deeper layers and may form a lipid layer that continues to reside in or on the stratum corneum after drying.[53]

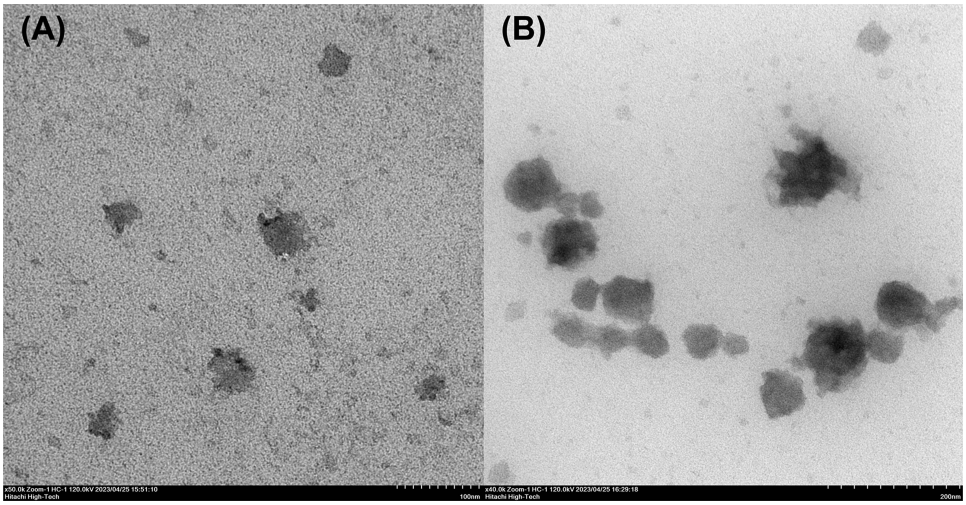

Figure 9 Transmission electron micrographs of nanoemulsion containing *G. bimaculatus* oil formulations F1 operating at 50k (**A**) and 40k (**B**) magnification.

All nanoemulsions from *G. bimaculatus* oil exhibited a narrow PDI between 0.125 ± 0.010 and 0.162 ± 0.013. Since PDI is generally used as an indication of their quality with respect to size distribution, the nano formulations with narrow PDI, particularly those smaller than 0.2, were defined as systems with homogeneous distribution.[53] Furthermore, all formulation exhibited the pronounced minus zeta potential values from −21.43 ± 2.08 to −28.23 ± 2.32 mV. Zeta potential is a fundamental parameter for electrokinetic potential for the thermodynamic stability of emulsions and colloidal systems, which represents the capacity of the particles to maintain a colloidal dispersion without coalescence, aggregation, or sedimentation.[54,55] With an increasing absolute magnitude of the zeta potential from zero, the repulsion between the suspended droplets increases, resulting in stable systems.[54]

The findings from this research noted that the concentration of the emulsifier system significantly affected the internal droplet size and PDI but not the zeta potential. Higher concentrations of the emulsifiers led to smaller internal droplet sizes but increased PDI. Regarding the small internal droplet size, narrow PDI, and pronounced zeta potential value, the nanoemulsions from *G. bimaculatus* oil were stable after the stability test in heating and cooling conditions as shown in Figure 8. Therefore, the nanoemulsions of *G. bimaculatus* oil, which were cosurfactant-free, were successfully developed. It was in line with the previous study showing that it is possible to fabricate the nanoemulsions from oil, water, and surfactant, which are the same ingredients as submicron emulsions.[56] However, the addition of cosurfactant would save on the high energy required by the homogenization. Among the three formulations developed in the present study, F1, which contained the smallest amount of emulsifier (2% w/w), was selected for further investigations on the in vitro cosmeceutical effects since it is an appealing formulation due to its small internal droplet size, significantly narrowest PDI, and significantly pronounced zeta potential.

Cosmeceutical Properties of Nanoemulsions from *G. bimaculatus* Oil

The cosmeceutical effects of F1, a nanoemulsion containing 1% w/w *G. bimaculatus* oil, were compared to the native oil (at the same concentration), as shown in Figure 10. The findings obviously noted that F1 had drastically enhanced cosmeceutical effects in terms of lipid peroxidation, anti-tyrosinase, and anti-ageing ($p < 0.001$). The findings were consistent with a previous study, which suggested that incorporating beneficial lipophilic bioactives into nanoemulsions provides a variety of benefits, notably higher antioxidant activity than the free form.[57]

Among various cosmeceutical activities, F1 was discovered to have extremely powerful anti-tyrosinase activities. In general, melanogenesis consists of two stages mediated by the tyrosinase enzyme, the first of which is DOPA synthesis and the second of which is DOPA oxidation to dopaquinone.[58] F1 was found to inhibit the tyrosinase activities when the substrates were both L-tyrosine and L-DOPA, with inhibitions of 96.5 ± 0.2% and 87.9 ± 1.4%, respectively. As tyrosinase is an enzyme that plays a part in the metabolic process that generates hyperpigmentation of the human skin via the biosynthesis of melanin in the human skin, the compounds or formulations that possessed anti-tyrosinase activity were appealing for whitening effects. F1 was thus an attractive formulation for skin whitening.

Aside from the antioxidant and whitening effects of F1, it also possessed a promising anti-ageing effect due to the significant inhibition of elastase (70.2 ± 2.3%), collagenase (46.3 ± 1.2%), and hyaluronidase (41.3 ± 0.6%), which was

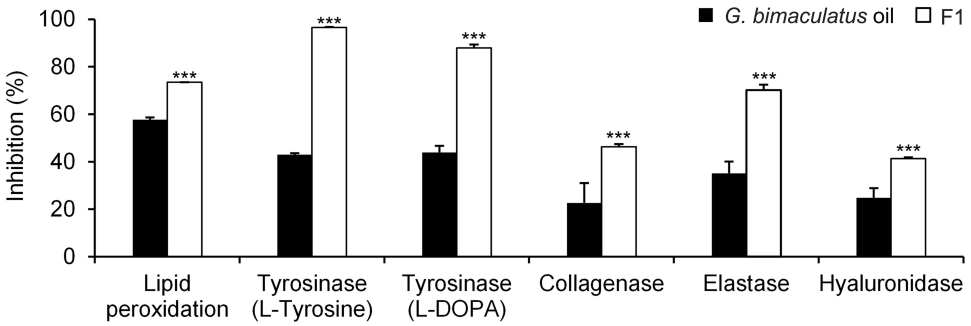

Figure 10 Inhibitory activities against lipid peroxidation, tyrosinase (when the substrate was L-tyrosine and L-DOPA), collagenase, elastase, and hyaluronidase of 1% w/w *G. bimaculatus* oil and nanoemulsion containing *G. bimaculatus* oil (F1). Asterisks (***) indicate a statistically significant difference between *G. bimaculatus* oil and F1 ($P<0.01$).

obviously elevated by incorporating *G. bimaculatus* oil in the nanoemulsion ($p < 0.01$). The likely explanations were due to the nanometric particle dispersion's outstanding reactivity and efficient transmission of bioactive molecules, which improved the biological activities of lipophilic compounds.[57,59]

Conclusion

G. bimaculatus yielded an oil that was rich in linoleic acid (31.08 ± 0.00%), oleic acid (30.44 ± 0.01%), palmitic acid (24.80 ± 0.01%), and stearic acid (7.61 ± 0.00%). The oil demonstrated promising cosmeceutical properties in terms of antioxidant, anti-tyrosinase, and anti-skin ageing activity, while causing no irritation in the in vitro HET-CAM test and cytotoxicity tests in HaCaT and Hs68 cells. A nanoemulsion of *G. bimaculatus* oil (F1) was successfully developed using only 2% w/w of the surfactant combination, with the HLB equal to the oil's RHLB, which was 9. Interestingly, nanoemulsion technology was found to be a powerful delivery system for enhancing the cosmeceutical attributes of *G. bimaculatus* oil. The nanoemulsion containing *G. bimaculatus* oil was proposed as an appealing cosmeceutical formulation with whitening, antioxidant, and anti-aging properties due to the significant improvements in the cosmeceutical properties of the *G. bimaculatus* oil. However, additional skin penetration experiments were recommended to be conducted to gain a more comprehensive understanding of the capacity of the nanoemulsion to penetrate the skin, that would enable a deeper understanding of the efficacy and performance of the nanoemulsion. Besides, further clinical testing was suggested to confirm the efficacy of the nanoemulsion.

Acknowledgments

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Disclosure

Dr Wantida Chaiyana reports a pending patent for cosmetic products containing cricket. The authors report no conflicts of interest in this work.

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