Fatty acid profile of Cobra Oil and its activities on skin melanoma cells

Suchitra Khunsap1, Supranee Buranapraditkun2, Panithi Laoungbua1 and Lawan Chanhome1

ABSTRACT

Cobra oil was used as a Thai traditional remedy to skin carefulness without scientific evidences. In this study, the oil was extracted from depot fat of Naja kaouthia; a monocled cobra by using a simple method. The oil components were detected by GC-MS which showed 30.34% of saturated fatty acid, 34.19% of mono-unsaturated fatty acid and 25.96% of polyunsaturated fatty acid. The main components were Palmitic acid (20.88%), Vaccenic acid (24.77%) and Linoleic acid (19.16%). Biological functions of Cobra oil were investigated for migration inhibition and apoptosis cell death on SK-MEL-28; human skin melanoma. Various concentrations of Cobra oils (20%, 10%, 5% and 2.5% v/v) could inhibit migration on SK-MEL-28 cells 91.43%±6.64%, 61.85%±10.61%, 26.80%±6.7% and 0% respectively. In addition, SK-MEL-28 cells which treated by Cobra oils (20%, 10%, 5% and 2.5% v/v) undergo apoptosis were detected using AnnexinV-FITC kit. The maximum oil concentration (10%) could induce 25% apoptosis in cells at 20 h and increased in concentration of oil was not increased apoptosis.

Key words: Polyunsaturated fatty acid, Apoptosis, wound healing, Cobra oil, Thai herb

1Corresponding author; E-mail address: sthaithumnas@yahoo.com
1Research of Development, Queen Saovabha Memorial Institute, Bangkok 10330, Thailand
2Cellular Immunology Laboratory Allergy and Clinical Immunology Unit, Department of Medicine, Faculty of Medicine Chulalongkorn University, Bangkok 10330, Thailand.
INTRODUCTION

Natural oils are wildly traditional remedies to skin carefulness and many diseases. The oil of plants and animals has been studied for pharmaceutical applications. Oil, extracted from many native crops, has been studied for pharmacological properties such as antimicrobial, (Nissen et al 2010) anti-inflammatory (Cardoso et al 2011), wound healing (Sünart et al 2011 and Sünart et al 2012) and also anti-cancer (Nikolakopoulou et al 2013). The active components of natural oils were concerned to polyunsaturated fatty acids which effected to many diseases such as cancers (Lim et al 2009, Mandal et al 2012 and Nikolakopoulou et al 2013).

Snake oils have been used as traditional medicine for remedy to skin problems, local tissue necrosis and wound assessments. The famous snake oil, Chinese water snake, was a rich source of eicosapentaenoic acid (EPA; omega-3 PUFA) which had potential to muscle, anti-inflammatory and confers therapeutic benefits (Kunin et al 1989). Fixed oil from Boa constrictor, the snake found in the riverine areas of Nigeria, was antimicrobial and anti-inflammatory (Falodun et al 2008). It had also shown 70% reducing actively proliferation on keloid fibroblasts of human skin (Olaitan et al 2011). In Thailand, Cobra oil has been used as a traditional remedy for wound healing, muscle pain, rheumatoid and also local tissue necrosis from animals bite or insects stings. Therefore, scientific studies should be performed to develop Thai herbal products for international market.

In this work, the fatty acid components of the monocled Cobra oil were determined by GC-MS analysis. The effects of Cobra oil were studied on skin melanoma cell line (SK-MEL-28). Wound healing assay was a tool for inhibition migration analysis. Apoptotic cells were detected using AnnexinV-FITC kit and measured by flow cytometry.

MATERIALS AND METHODS

1. Materials

The fat sack was excised from the monocled Cobra (Naja kaouthia) and stored at -20 ⁰C. MEM medium, FBS (Fetal bovine serum), streptomycin, penicillin were purchased from Sigma (Gibco, USA). Other chemicals were analytical grade.

2. Lipids extraction

Lipid from snake’s fat sack was extracted by simply method. The fat sack was cut into small pieces and put on a petri-dish and incubated 37 ⁰C until cells became shrunken. The lipid was kept at -20 ⁰C until used.
3. Fatty acid profile analysis (FAMEs: Fatty acid methyl ester)

Two hundred microliters of lipid was simultaneously transesterified with 0.3 ml 5% HCl/MeOH and incubated at 85 °C for 1 hr. The sample was dissolved with hexane and detected by using a gas chromatography coupled to a mass spectrometer (GC/MS). The injector and detector were 250 °C split mode. One hundred microliters of sample was injected into an Innowax column (30m x 0.25 mm, film thickness 0.25 µm). The carrier gas was helium at flow rate 1.0 ml/min. The oven temperature was performed from 50 °C to 200 °C at 20 °C/min and followed by 230 °C at 3 °C/min. The detector was recorded at 30 to 400 amu. The Wiley database was used as standard components for the spectrometer.

4. Cell cultures

SK-MEL-28 cell was supplemented with 10% FBS (Fetal bovine serum) in MEM medium plus 1 mM glutamine, 100U/ml streptomycin and 100U/ml penicillin. Cells were incubated in a 5% CO₂ chamber 37 °C.

5. Migration inhibition

Wound healing assay was used for detecting migration inhibition. Cell line, 5x10⁵ cells/ml, was seeded in 24 well plates and incubated at 37 °C with 5% CO₂ for 24 hours. The concentrations of snake oil (20, 10, 5 and 2.5 %; v/v) was added into the cells and incubated at 37 °C with 5% CO₂ for 24 hours. Migration inhibition was determined by measuring the distance of the edge scratching. The equation of migration inhibition was 100-[(Z-Tn)/Z] x100. Z is the negative distance at time 0. Tn is the experiment distances at time 24 h.

6. Apoptotic analysis

Cell line was seeded in 6 well plates and incubated at 37 °C for 24 hours. The various optimal concentrations of snake oil (20, 10, 5, 2.5 and 0% v/v) were added into the cells and incubated at 37 °C with 5% CO₂ for 20 hours, harvested and washed with PBS 7.2. Cell was stained with AnnexinV-FITC and propidium iodide (Santa Cruz Biotechnology, Inc) for 15 min. Apoptotic cell was detected by flow cytometry.
7. Statistical analysis

Results were compared using t-test (PRIMER of Biostatistics version 3.02). Differences between the cells which untreated (0% oil) and treated (various concentration of % oils) were considered significant at $p<0.05$. The picture of migration inhibition represented as mean ± SD whereas the picture of apoptosis analysis and fatty acid components shown as mean ± SEM.

RESULTS AND DISCUSSIONS

1. Lipid extraction and lipid profile of Cobra oil

In this study, lipid was extracted by the very simple method which not Soxhlet, Bligh & Dyer and Folch. The depot fat of snake was cut and blended, then incubated at 37°C until lipid came out. This method was easy, no extra instruments and specialist were needed. However, fatty acid compositions of Cobra oil has been similarly to the lipid profile of Spilotes pullatus snake which extracted by Soxhlet apparatus (Oliveira et al. 2014). Lipid components of Cobra oil consisted of 30.34% saturated, 34.19% of monounsaturated and 25.96% of polyunsaturated fatty acids. The results represented 20.88% of Palmitic acid, 24.77% of Vaccenic acid and 19.16% of Linoleic acid as the major of saturated, monosaturated and polysaturated fatty acids respectively (Table1). Palmitic acid found the main saturated fatty acid of various reptile lipids while unsaturated fatty acid was difference (Ackman et al. 1991; Buthelezi et al. 2012 and Oliveira et al. 2014). It might be species different or depended on ecological conditions. For example, fatty acid profile of the monocled Cobra oil had similarly to Spilotes pullatus Linn, a Cobra snake in Brazil (Oliveira et al. 2014).

However, Cobra oil contained essential fatty acids which worked against microbial (Hammer et al. 2008), cancers (Caldefie-Chézet et al. 2006; Mandal et al. 2012) and other diseases (Gil, 2002). The ratio and fatty acid interaction should be further studied.
Table 1. Fatty acid components extracted from the monocled Cobra oil by the simple method.

<table>
<thead>
<tr>
<th>Fatty acid components</th>
<th>% Quality</th>
<th>% Area</th>
<th>SEM</th>
<th>RT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undecylic acid</td>
<td>95</td>
<td>0.21</td>
<td>0.04</td>
<td>8.35</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>98</td>
<td>0.88</td>
<td>0.09</td>
<td>9.60</td>
</tr>
<tr>
<td>Pentadecylic acid</td>
<td>93</td>
<td>0.24</td>
<td>0.02</td>
<td>10.34</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>99</td>
<td>20.88</td>
<td>1.63</td>
<td>11.23</td>
</tr>
<tr>
<td>Margaric acid</td>
<td>97</td>
<td>0.45</td>
<td>0.03</td>
<td>12.21</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>99</td>
<td>4.97</td>
<td>0.45</td>
<td>13.43</td>
</tr>
<tr>
<td>Arachidic acid</td>
<td>92</td>
<td>0.28</td>
<td>0.00</td>
<td>16.31</td>
</tr>
<tr>
<td>Palmitinic acid</td>
<td>99</td>
<td>2.44</td>
<td>0.73</td>
<td>21.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>30.34</strong></td>
</tr>
<tr>
<td><strong>Monounsaturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>99</td>
<td>5.46</td>
<td>0.49</td>
<td>11.49</td>
</tr>
<tr>
<td>Vaccenic acid</td>
<td>99</td>
<td>24.77</td>
<td>2.30</td>
<td>13.74</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>99</td>
<td>3.96</td>
<td>0.07</td>
<td>13.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>34.19</strong></td>
</tr>
<tr>
<td><strong>Polyunsaturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic acid (LA)</td>
<td>99</td>
<td>19.16</td>
<td>1.90</td>
<td>14.37</td>
</tr>
<tr>
<td>α-Linolenic acid (ALA)</td>
<td>99</td>
<td>1.56</td>
<td>0.00</td>
<td>15.26</td>
</tr>
<tr>
<td>Eicosatrienoic acid (ETE)</td>
<td>90</td>
<td>0.57</td>
<td>0.03</td>
<td>17.95</td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>91</td>
<td>4.68</td>
<td>0.88</td>
<td>25.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>25.96</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>90.49</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pheromone, Cholesterol

2. Migration inhibition by wound healing assay

Migration inhibition on SK-MEL-28 cell was induced by Cobra oil depended on doses manner. Various doses of Cobra oil (20%, 10%, 5%, 2.5% and 0%, v/v) could inhibit the proliferation of SK-MEL-28 cells; 91.43%±6.64%, 61.85%±10.61%, 26.80%±6.7% and 0% respectively, for 24 h (Fig.1). The percent inhibition was not increasing even if using higher doses then. The 20% (v/v) of Cobra oil was strongly inhibited on SK-MEL-28 cell which equaled to Drs-PLA₂ from Daboia siamensis venom (Khunsap et al 2011). The component of Cobra oil could effect to the cells clear. For example, omega-3 fatty acid was reported in the prevention of non-melanoma skin cancer (Black et al 2006)
and also regulated breast tumor via miR-21 (Mandal et al 2012). In contrast, both (n-3) and (n-6) fatty acids could stimulate wound healing in the rat intestinal epithelial cell line, IEC-6 (Ruthing et al 1999). Moreover, Linoleic and Oleic acids were repaired would tissue of BALB/mice skin (Cardoso et al 2011). Probably, the effects of fatty acids depended on source, ratio of components and cell type.

![Figure1](image)

**Figure1.** Migration inhibition on SK-MEL-28 cell was induced by the monocled Cobra oil. Result showed as mean ± SD (n=6). Differences between the cells which untreated (0% oil) and treated (2.5, 5, 10 and 20% oils) were considered significant at *p<0.05.

3. **Apoptotic cell analysis**

Twenty five percent was the highest apoptosis which induced by Cobra oil (10%, v/v) for 20 h. Two point five (% , v/v) of Cobra oil induced the lowest apoptosis which closed to untreated cells (Fig2). Increasing concentration of Cobra oil was not induced more apoptosis (data not shown). The result indicated that Cobra oil had limited effects to apoptotic on SK-MEL-28 cells. It so probable that Cobra oil effected to the other cell death programs. The Cobra oil has high potential induced apoptosis on cancer cell when compared with only docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) (Nikolakopoulou et al 2013). However, the optimal incubation time and the other program of cell death need to study further.
Figure 2. Total apoptosis of SK-MEL-28 was induced by the monocled Cobra oil for 20 h. Data represented as mean ±SEM (n=6). Differences between the cells which untreated (0% oil) and treated (2.5, 5, 10 and 20% oils) were considered significant at *p<0.05).

CONCLUSION
This was an initial study of Thai traditional herb, the monocled Cobra oil. The oil was extracted by a simple method which might be provided easily by people in remote areas. The oil had potential inhibited migration and slightly induced apoptotic cell death on SK-MEL-28 cells. The other studies are going on processes.

ACKNOWLEDGEMENTS
This work was supported by Queen Saovabha Memorial Institute, Thai Red Cross Society. We thankful Sunutchta Suntrarachun and Jureeporn Noiporn for revised the manuscript and statistical analysis.


Mandal C.C, Ghosh-Choudhury T, Dey N, Ghosh-Choudhury G and Ghosh-Choudhury N. 2012. miR-21 is targeted by Omega-3 polyunsaturated fatty acid to regulate breast tumor CSF-1 expression.


Ruting D.J and Meckling-Gill K.A. 1999. Both (n-3) and (n-6) fatty acids stimulate wound healing in the rat intestinal epithelial cell line, IEC-6. Biochemical and molecular action of nutritions. 1791-1798.
