5. Conclusion

The method for quantifying total polyphenol content in *Terminalia catappa* L. leaves was developed by using UV-Vis spectroscopy and the coloring reaction between the sample solution and Folin-Ciocalteu reagent before photometric measurement at wavelength 730 nm. The method was validated to show good linearity (R^2 = 0.9957), precision (% relative standard deviation < 2%), accuracy (92.28% - 93.64%), limits of detection (0.033 µg/ml) and quantification (0.100 µg/ml). The results of analyzing 10 leaf samples performed total polyphenol content ranging from 53.62 to 65.46 mg GAE/g.

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EFFICACY OF DIHYDROQUERCETIN NANOEMULSION IN TYLOXAPOL-INDUCED HYPERLIPIDEMIC MICE

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Summary

Efficacy of Dihydroquercetin Nanoemulsion in Tyloxapol-Induced Hyperlipidemic Mice

Dihydroquercetin (DHQ), also called as taxifolin, has been reported to have many beneficial properties for human health. However, its low solubility and bioavailability are major obstacles for the biomedical application of the flavonoid. This study aims to evaluate the effects of dihydroquercetin (DHQ) and dihydroquercetin loaded self-nano-emulsifying drug delivery systems (NanoDHQ) containing 8% DHQ on lipid profiles in tyloxapol-induced hyperlipidemic mice. *Swiss albino* mice were daily treated with DHQ (24 and 48 mg/kg, p.o) or NanoDHQ (37.5, 75 and 150 mg/kg) or fenofibrate (100 mg/kg, p.o), a reference drug, two week before tyloxapol injection (Triton WR 1339, 250 mg/kg, i.v). After 16-hour administration of tyloxapol, blood was collected and the levels of serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C), non-high-density lipoprotein-cholesterol (non-HDL-C), and low-density lipoprotein-cholesterol (LDL-C) were measured. Our results showed that the levels of TC, TG, LDL-C, non-HDL-C in the model group were significantly increased, while the HDL-C level of the animals were markedly decreased in comparing with the control group. The treatment of DHQ at the dose of 48 mg/kg significantly decreased TG level but not altered the other lipid indexes as compared to the model group. Interestingly, the administration of NanoDHQ (75 and 150 mg/kg) significantly and dose-dependently attenuated the elevation of TC and TG level in the tyloxapol-treated mice. Moreover, the NanoDHQ treatment

ameliorated the reduction of HDL-C level and the elevation of non-HDL-C level in the Tyloxapol-treated animals. NanoDHQ treatment had no significant effect on LDL-C levels of the animals. In conclusion, the present study demonstrated that the administration of NanoDHQ enhanced the efficacy of DHQ on hypolipidemic activity in tyloxapol-induced hyperlipidemic mice. NanoDHQ treatment may be useful for treatment of hyperlipidemia.

Keywords: *Dihydroquercetin, Taxifolin, Dihydroquercetin nanoemulsion, Hypolipidemic activity.*

1. Introduction

Hyperlipidemia is generally considered to be a major risk factor for cardiovascular diseases such as atherosclerosis. Hyperlipidemia is characterized by abnormal elevation of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) [1]. In the United States, cardiovascular disease is the leading cause of death among adults, and people with hyperlipidemia are at roughly twice the risk of developing cardiovascular disease as compared to those with normal total cholesterol levels. Treatments of these disorders include diet control, physical exercise, surgery, and medications [2]. Currently, although statins, fibrates and nicotinic acid derivatives,.. have been widely used to reduce plasma lipids. However, their usage may be limited due to their side effects such as hepatotoxicity, rhabdomyolysis, or skeletal muscle injury [3]. Thus, alternative therapeutics using natural products have been proposed to control lipid metabolism.

Among bioactive compounds derived from plants, flavonoids have been identified to be the most beneficial in terms of alleviation of human diseases. Dihydroquercetin (DHQ) is 3,5,7,3′,4′-pentahydroxyflavanone or taxifolin, an isoflavanone in flavonoids family. It is found in plants of various families but is isolated in large amounts (up to 4.5%) only from Siberian larch (*Larix sibirica*) or Dahurian larch (*L. gmelinii*) [4]. The flavonoid gained the attention for its large pharmacological actions including anti-oxidant, anti-inflammatory, hepatoprotective, anti-Alzheimer, anti-angiogenic, cardiovascular activity, anti-microbial activity and anti-viral activity [5]. However, on account of the slight solubility of DHQ, it was difficult to be absorbed and metabolized by the body, which greatly limited its bioavailability and efficacy [6].

Various methods for increasing the solubility and bioavailability of DHQ have been described in the literature, in which nanotechnology development plays a significant part of the corresponding research over the last decade. In 2014, Zu et al. prepared taxifolin nanosuspension of 24.6 nm optimized particle size using the liquid antisolvent precipitation method with ethanol as solvent, deionized water as antisolvent and poloxamer 188 as the selected surfactant [6]. The experimental data showed that the solubility and dissolution rate of taxifolin nanoparticles are 1.72 and 3 times higher than those of raw taxifolin, respectively. The taxifolin nanoparticles also displayed superior antioxidant capcity and increased the bioavailability by 7 times compared to raw taxifolin. Another article published in 2016 by Yang et al. employed a dissolution-fusion method with polyvinylpyrrolidone as drug carrier to produce taxifolin nanodispersion [7]. Pharmacokinetics analysis using UHPLC-MS/MS method calculated the absolute bioavailability of taxifolin nanodispersion to be 0.79% while that of raw taxifolin is 0.49%. Terekhov et al. synthesized DHQ lyophilizates with mixtures of water and ethanol/acetonitrile as solvents [8]. Results of dissolution testing for the obtained lyophilizates demonstrated that lyophilization increases water solubility of DHQ by at least 30 times while keeping the antioxidant activity of initial DHQ intact.

In this study, we investigated the effects of DHQ and NanoDHQ on lipid profiles using tyloxapol-induced hyperlipidemic mice. The present finding provided scientific evidences for the efficacy of NanoDHQ on hyperlipidemia.

2. Materials and Methods

2.1. Chemicals and reagents

Dihydroquercetin powder (DHQ content ≥ 95%, trade name: Flavitol) extracted from Siberian larch (*Larix sibirica*) was purchased from Russia. Soybean lecithin 99% of pharma grade was obtained from Parul Trading Co., India. Polyethylene glycol (PEG-400), absolute ethanol 99.5% and polysorbate 80 (Tween 80) were purchased from Sigma- Aldrich.

For pharmacological study, tyloxapol (Triton WR 1339) was purchased from Sigma. Fenofibrate (Trade name: Lipanthyl) was purchased from Abbott Indonesia. Total cholesterol (TC), total triglyceride (TT) and high-density lipoprotein-cholesterol (HDL-C) diagnostic kits were obtained from EBRA (EBRA chem, India).

2.2. Animals

Both male and female *Swiss albino mice* of 4-5 weeks old weighing between 18-20 grams were provided by the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. Mice were housed in the laboratory animal room of National Institute of Medicinal Materials (25 ± 1°C under 65 ± 5% humidity and 12 h dark-light cycle (from 7:00-19:00)). Food and water were given *ad libitum*.

Mice were kept for one week to acclimatize before the commencement of experiments.

2.3. Preparation of DHQ nanoemulsions (NanoDHQ)

Dihydroquercetin loaded self-nano-emulsifying drug delivery systems (SNEDDS) was prepared at Institute of Physics, Vietnam Academy of Science and Technology with following 3 steps [9]:

Step 1: DHQ powder (9.5 g) was dissolved in 200 mL absolute ethanol in a 500 mL glass beaker. The solution was slowly heated to 60ºC and stirred using a magnetic stirrer for 60 min until perfect dissolution. Soybean lecithin of 15 g was then added to the above solution, this mixture was continuously stirred for 60 min, and finally treated with ultrasonic vibration for 60 min. The resulting mixture was called Solution A.

Step 2: A 85 g composition consisting of Tween 80, PEG and distilled water was mixed together at a weight ratio of 2:1:1. The mixture slowly heated up to 60ºC and stirred using a magnetic stirrer for 30 minutes to create a homogeneous system, called Solution B.

Step 3: Solution B was poured into solution A. The mixture was immediately stirred and slowly heated up to 80ºC with a rate of 2ºC/min. The heating was continued until the temperature reached and the stirring was remained for more than 4 hrs in order to form a brown gel-like homogeneous mixture. During the preparation, the SNEDDS performance of the mixture was visually checked over time by dilution with pure water until a clear solution was observed. The mixture was stored at room temperature until used.

The analysis showed that the DHQ nanoemulsion with 8% DHQ content was successfully synthesized with typical mean nanoparticle sizes from 9 to 11 nm. The nanoparticle size was determined by dynamic light scattering (DLS - Zetasizer Nano-S90 technique of Malvern Instruments, UK). The mixture was stored at room temperature until used.

2.4. Animal model and treatments

The hypolipidemic activity of DHQ and NanoDHQ were evaluated using tyloxapol-induced hyperlipidemic model in mice according to a previous description [10]. The dose of DHQ and NanoDHQ for testing the hypolipidemic activity were selected through preliminary experiments.

* For evaluation the effect of DHQ, mice were divided into 5 groups with 9-10 mice (5 male mice) per group as below:

- Group 1 (Control): distilled water 10 mL/kg/day (p.o)
- Group 2 (Model): Tyloxapol injection and distilled water 10 mL/kg/day (p.o).
- Group 3 (DHQ 24 mg/kg): Tyloxapol injection and DHQ at the dose of 24 mg/kg/day (p.o).
- Group 4 (DHQ 48 mg/kg): Tyloxapol injection and DHQ at the dose of 48 mg/kg/day (p.o).
- Group 5 (Fenofibrate): Tyloxapol injection and Fenofibrate at the dose of 100 mg/kg/day (p.o).

* For evaluation the effect of NanoDHQ, mice were divided into 6 groups with 9-10 mice (5 male mice) per group as below:

- Group 1 (Control): distilled water 10 mL/kg/day (p.o)
- Group 2 (Model): Tyloxapol injection and distilled water 10 mL/kg/day (p.o).
- Group 3 (NanoDHQ 37.5 mg/kg): Tyloxapol injection and NanoDHQ at the dose of 37.5 mg/kg/day (p.o).
- Group 4 (NanoDHQ 75 mg/kg): Tyloxapol injection and NanoDHQ at the dose of 75 mg/kg/day (p.o).
- Group 5 (NanoDHQ 150 mg/kg): Tyloxapol injection and NanoDHQ at the dose of 150 mg/kg/day (p.o).
- Group 6 (Fenofibrate): Tyloxapol injection and Fenofibrate at the dose of 100 mg/kg/day (p.o).

Mice were administered with DHQ or NanoDHQ or fenofibrate, a reference drug two weeks before injection of tyloxapol (250 mg/kg, i.v). After 16 hours administration of tyloxapol, 300 ml blood from cervical artery was collected to measure serum TC, TG, HDL-C using Biochemical analyzer (Humanlyser, Germany) with commercial ERBA diagnostic kits. Low-density lipoprotein-cholesterol (LDL-C) level was calculated using the formular: LDL-C= (non-LDL-C- TG)/5. Non-LDL-C concentration was calculated using the formula: non-LDL-C=TC−(HDL-C) [11].

2.5. Statistical analysis

SigmaPlot 12.0 (SYSTA Software Inc, Richmond, CA, USA) was used for statistical analysis. Data are expressed as the mean ±S.E.M. and analyzed by unpaired Student's t-test for two groups or one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparison. p values < 0.05 were considered statistically significant.

3. Results

3.1. Effects of DHQ on serum lipid profile of tyloxapol-induced hyperlipidemic mice

Tyloxapol-induced hyperlipidemic mice were employed to evaluated the lipid lowering effect of DHQ. As shown in Fig. 1, the concentration of TC, TG in the model group were significantly increased in comparing with the control group.

The treatment of DHQ at the dose of 48 mg/kg significantly decreased of serum TG but not TC concentration of the mice compared to the model group. At the lower dose, DHQ had no significance effect on TC as well as TG. Fenofibrate at the dose of 100 mg/kg/day markedly decreased the tyloxapol-induced the elevation of TC and TG in mice.

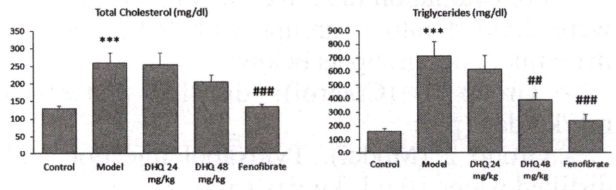

Fig. 1. Effects of DHQ extract on total cholesterol (TC) and triglycerides (TG) concentration in tyloxapol-induced hyperlipidemic mice.
*Data were presented as the mean ± S.E.M. (n=9-10). ***p<0.001 vs. control group; ##p< 0.01, ###p <0.001 vs model group.*

As shown in Fig. 2, HDL-C concentration in the model group was significantly decreased in comparing with the control group. In the other hand, the concentration of non-HDL-C and LDL-C in the model group animals were markedly increased comparing to the control mice. Treatment of DHQ at the dose of 24 mg/kg, as well as 48 mg/kg had no significant effect on these lipid indexes. Fenofibrate at the dose of 100 mg/kg reversed the tyloxapol-induced the change of HDL-C, non-HDL-C and LDL-C levels in mice.

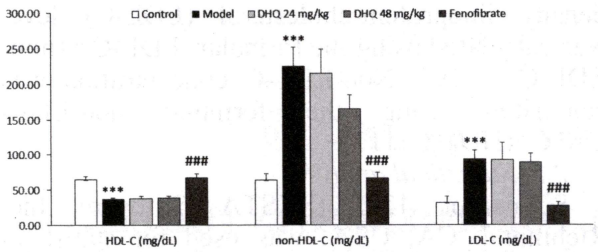

Fig. 2. Effects of DHQ on HDL-C, Non-HDL-C and LDL-C concentrations in tyloxapol-induced hyperlipidemic mice.
*Data were presented as the mean ± S.E.M. (n=9-10). ***p<0.001 vs. control group; ###p <0.001 vs model group.*

3.2. Effects of NanoDHQ on serum lipid profile of Tyloxapol-induced hyperlipidemic mice

The hypolipidemic effects of NanoDHQ was also evaluated using the tyloxapol -induced hyperlipidemia in mice. As shown in Fig 3, NanoDHQ at the dose of 37.5 mg/kg had no significant effect on the TC and TG levels of the hyperlipidemic mice. Administration of NanoDHQ at the dose of 75 mg/kg significantly decreased the levels of TG but not TC in the tyloxapol-treated mice. Interestingly, NanoDHQ treatment at the dose of 150 mg/kg significantly reduced both TC and TG indexes.

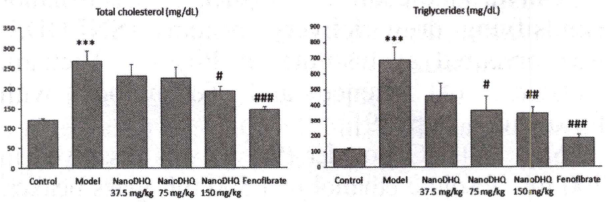

Fig. 3. Effects of NanoDHQ extract on total cholesterol (TC) and triglycerides (TG) concentration in tyloxapol-induced hyperlipidemic mice.
*Data were presented as the mean ± S.E.M. (n=9-10). ***p<0.001 vs. control group; #p< 0.05, ##p< 0.01, ###p <0.001 vs model group.*

Fig. 4 illustrated the effects of NanoDHQ on the concentration of HDL-C, non-HDL-C and LDL-C in the hyperlipidemic animals. NanoDHQ treatment had no significant effect on the LDL-C levels of the mice. However, NanoDHQ at the doses of 75 and 150 mg/kg reversed the decrease of HDL-C in the Tyloxapol-treated animals. At the dose of 150 mg/kg, the NanoDHQ treatment showed a significant effect on the Tyloxapol - induced the elevation of non-HDL-C index.

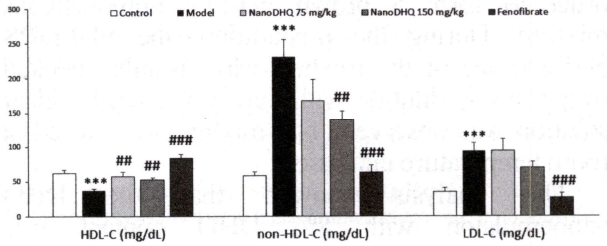

Fig. 4. Effects of NanoDHQ on HDL-C, Non-HDL-C and LDL-C concentrations in tyloxapol-induced hyperlipidemic mice.
*Data were presented as the mean ± S.E.M. (n=9-10). ***p<0.001 vs. control group; #p< 0.05, ##p< 0.01, ###p <0.001 vs model group.*

4. Discussion

In this study, we employed tyloxapol-injected mice, an animal model of endogenous dyslipidemia to evaluate the hypolipidemic efficacy of DHQ and NanoDHQ. Our results showed that a single dose (250 mg/kg) of tyloxapol induced the marked elevation of serum TC, TG, non-HDL, LDL and decreased serum HDL after 16 hrs i.v injection in mice. The nonionic detergent tyloxapol (Triton WR-1339) produces hyperlipidemia because the inhibition of lipoprotein lipase activity, produces an elevation of plasma LDL with a rapid decrease in

HDL without altering the albumin concentration. The model widely used for screening the hypolipidemic agents because of the rapid and high reproducible [12],[13]. Using this hyperlipidemic model, we demonstrated that treatment of DHQ at the dose of 48 mg/kg significantly decreased of TG level but not altered the other lipid indexes in the blood of tyloxapol-treated mice. In previous report, the anti-hyperlipidemic activity of DHQ has been evaluated using cholesterol rich diet-fed rats, an animal model of exogenous dyslipidemia. Results showed that the DHQ-supplemented diet maintains the normal lipid profile in the serum and liver, and lipid excretion in fecal of the hyperlipidemic rats [14]. Thus, our finding suggested that the treatment of DHQ may bring some beneficial actions for modulation of lipid profiles of the hyperlipidemic animals.

In the present study, we demonstrated that the treatment of NanoDHQ (75 and 150 mg/kg) with 8% DHQ content, significantly and dose-dependently attenuated the elevation of TC and TG level in the hyperlipidemic mice. Moreover, the NanoDHQ treatment attenuated the reduction of HDL-C level and the elevation of non-HDL-C level in the animals. To date, this is the first report demonstrated the anti-hyperlipidemic effects of NanoDHQ using an animal model of hyperlipidemia. In this study, though nanoDHQ had no significant effect on LDL-C, but the nano emulsion showed a tendency to reduce the lipid index in the tyloxapol-injected mice. Therefore, a higher dose of the nanoDHQ should be tested in future study using diet-induced a hypercholesterolemia model which is the most relevant stimulus for the induction of atherosclerotic lesions in humans.

The present finding indicated that NanoDHQ is superior to DHQ on hypolipidemic activity. Recently, using *in vitro* experiments on antioxidant activities against ABTS radical, our group demonstrated that the NanoDHQ was also stronger than the raw DHQ as well as the control Trolox with the IC_{50} values of NanoDHQ, DHQ, and Trolox were 238.5, 474,2 and 331 (μg/ml), respectively [9]. It is widely known that the circulation time of orally administered drugs is reduced because of the first-pass mechanism in liver and they can be quickly cleared by the gastrointestinal tract. Encapsulation of the drug molecules in nano-based delivery systems prolongs the release of drug inside the body. Furthermore, the small size of nanoparticles allows them to cross the cell membranes *via* endocytosis. The amphiphilic drug carriers also increase absorption of hydrophobic drugs in the intestine [15]. An examplary study by Yang et al. on Wistar rats compared the pharmacokinetic parameters of taxifolin nanodispersion and its physical mixture in rat plasma. The AUC_{0-t} from mean plasma concentration vs time plot of taxifolin nano dispersion (90.89 ± 11.76 ng h/mL) is significantly higher than that of the physical mixture of taxifolin after oral administration (59.11 ± 8.62 ng h/mL), which translates to a higher value of absolute bioavailability [7]. All above mentioned factors may explain the improved efficacy of NanoDHQ in comparison with that of raw DHQ. Taken together, the present study indicated that the nano-based delivery systems may be useful for improving the bioavailability and efficacy of DHQ. Thus, beside anti-oxidant and anti-hyperlipidemic effects, further investigation should be done to evaluate the anti-dementia, anti-angiogenic and neuroprotective effects of the NanoDHQ since its raw DHQ exerted these valuable pharmacological actions [5].

5. Conclusion

In conclusion, the present study demonstrated that the administration of NanoDHQ enhanced the efficacy of DHQ on hypolipidemic activity in tyloxapol-induced hyperlipidemic mice. Our finding suggested that NanoDHQ treatment may be useful for treatment of hyperlipidemia.

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EFFECTS OF *n*-BUTANOL EXTRACT FROM *VITEX TRIFOLIA* FRUITS ON COGNITIVE FUNCTION DEFICIT IN OVARIECTOMIZED MICE

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Summary

Effects of *n*-Butanol Extract from *Vitex trifolia* Fruits on Cognitive Function Deficit in Ovariectomized Mice

While changes in cognition may occur with the menopausal transition, clinical trial data have not demonstrated that treatment with postmenopausal hormone treatment prevents development of dementia or cognitive impairment. *Vitex trifolia* L. (VT) has been used as a herbal medicine for headaches and anti-depressants in menopausal women. In the present study, we have evaluated the effect of *n*- BuOH extract from the fruit of VT (30 and 60 mg/kg) treatment on cognitive impairment, bone index, and uterine atrophy in ovariectomized (OVX) mice. Five groups of female mice were used; sham group, OVX + water, OVX + VT extracts (30 and 60 mg/kg), and OVX + Progynova® (estradiol valerate, 0.5 mg/kg). Our data revealed that VT *n*-BuOH extract significantly mitigated memory decline and antagonized the atrophy of the uterus in OVX mice. We also found that VT *n*-BuOH extract markedly increased the serum level of estradiol, suggesting that VT *n*-BuOH extract relieves the symptoms of menopause through, at least in part, stimulating the biosynthesis of estrogen in circulation. Taken together, our results demonstrate that *n*-BuOH extract from the fruit of VT might be beneficial for treatment of cognitive deficits in postmenopausal women.

Keywords: *Vitex trifolia* L., *Ovariectomized mice, Cognitive deficit, Uterus-oviduct, Estradiol, Alkaline phosphatase.*

1. Introduction

Postmenopausal hormone therapy is used for controlling menopausal symptoms and it provides relief for uncomfortable symptoms of menopause such as hot flashes and night sweats, reduces bone loss and risk of fractures, and (administered locally) addresses genitourinary syndrome of menopause [1] but has no demonstrable benefit for cognition [2].

Vitex trifolia L. (Verbenaceae) (VT) is a tropical shrub or shrubby plant that is widely distributed in Southeast Asia, Micronesia, Australia, and East Africa [3]. VT has been used as a part of traditional medicine for treatment of a wide range of diseases, including fever, headache, diarrhea, and tuberculosis [4],[5]. Moreover, the results from our previous study revealed that the ethanol extract of the fruits of VT improves learning deficits in ovariectomized mice by using the open field test and Morris water maze task [6]. Interestingly, our recent premilinary data showed that *n*-BuOH extract of VT fruits contains agnuside, which is demonstrated to exert estrogenic activity, with yeild higher than that in ethanol extract (4.05% and 0.37%, respectively). This raises a possibility that VT *n*-BuOH extract might also be beneficial for relieving the uncomfortable symptoms of postmenopause. Therefore, in the present study, we have further evaluated the activity of the *n*-butanol extract from the fruits of VT on the memory function as well as on uterus, bone, and