151-156. **4.** TCVN 10916:2015, Foodstuffs - Determination of minerals in infant formula and enteral products-atomic absorption spectrophotometric method. **5**. AOAC 985.35 (1988), Minerals in Infant Formula, Enteral Products and Pet Foods. Atomic Absorption Spectrophotometric Method. **6.** Imas Solihat, Didah Nur Faridah, Nancy Dewi Yuliana, Molekul (2018), Validation Method of Flame-AAS with Microwave Digestion for Mineral Analysis in Carbohydrate - Rich Samples, *Molekul*, 13 (2), 133-140. **7.** Nabil Ramadan Bader (2011), Sample preparation for flame atomic absorption spectroscopy: an overview, *Rasayan Journal of Chemistry*, 4(1), 49-55. **8.** Maria das G. A. K., Elane S. da B. M., Daniele C. M. B. dos S., Jacira T. C.; José T. P. B., Alete P. T., Andrea P. F., Bernhard W., Wagna P. C. dos S., Eduardo B. G. N. dos S., Mauro K. (2008), Sample Preparation for the Determination of Metals in Food Samples Using Spectroanalytical Methods-A Review, *Applied Spectroscopy Reviews*, Taylor & Francis Publisher, 43, 67-92. **9.** Ministry of Health (2018), Vietnamese Pharmacopoeia V. Medicine Publisher, episode 2, PL 124-125, PL 203. **10.** Ferreira S. L. C., Bruns R. E., Ferreira H. S., Matos G. D., David J. M., Brandao G. C., Silva E. G. P., Portugal L. A., Reisc P. S., Souza A. S., Santos W. N. L. (2007), Box-Behnken design: An alternative for the optimization of analytical methods, *Analytica Chimica Acta*, 597(2), 179-186. **11.** International Conference on Harmonization (ICH) (2005), Validation of Analytical Procedures: Test and Methodology, 1-15. **12.** Association of Official Analytical Chemists (AOAC) Guidelines for Single Laboratory (2002), Validation of Chemical Methods for Dietary Supplements and Botanical, 18-19.

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STANDARDIZED FLAVONOID EXTRACT FROM *DIOSPYROS KAKI* L.F LEAVES IMPROVES DYSLIPIDEMIA IN HIGH-CHOLESTEROL DIET FED RATS

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Summary

Standardized Flavonoid Extract from *Diospyros kaki* l.f Leaves Improves Dyslipidemia in High-Cholesterol Diet Fed Rats

The current study designed to investigate the anti-dyslipidemia effects of the standardized flavonoid extract from *Diospyros kaki* L.f leaves (DK extract) in chronic high-cholesterol diet fed rats. Wistar rats were orally administered oil-cholesterol mixture. Rats were daily treated with DK extract (50 and 100 mg/kg of body weight; p.o.) or atorvastatin (10 mg/kg of body weight; p.o.) for 4 consecutive weeks. Body weight, serum lipid profiles, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were evaluated for every 2 weeks. Body weight was significantly increased in the DK extract-treated group compared to the vehicle-treated group. Treatment of DK extract decreased the serum total cholesterol, triglycerides, and non-high-density lipoprotein cholesterol levels, and increased the serum high-density lipoprotein cholesterol. Additionally, DK extract substantially diminished enzymatic activity serum AST and ALT which were increased in the hypercholesterolemia rats. Our finding suggested that DK extract improves dyslipidemia and liver function in high-cholesterol diet fed rats.

Keywords: *Diospyros kaki* L.*f leaves, Flavonoid, High-cholesterol diet, Dyslipidemia.*

1. Introduction

Dyslipidemia is a major contribution to the onset of cardiovascular diseases such as atherosclerosis, coronary heart disease, and cerebrovascular disease, which are the main causes of the global burden of diseases [1]. Dyslipidemia is defined as elevation of serum total cholesterol and/or triglyceride or reduced high-density lipoprotein cholesterol. Treatments of these disorders include diet control, physical exercise, surgery, and medications [2]. Currently, although statins have been widely used to reduce plasma lipids, their usage may be limited due to their side effects such as hepatotoxicity, rhabdomyolysis, or skeletal muscle injury [3]. Thus, alternative therapeutics using herbs and/or natural products have been proposed to control lipid metabolism [4].

Diospyros kaki L.f., called persimmon, belongs to the family Ebenaceae. This plant is widely distributed in China, India, Japan, Korea, and Vietnam. Persimmon leaves were traditionally utilized as a medicine, health beverage, and cosmetic [5]. Evidence showed that the powdered whole persimmon leaf improved plasma and hepatic lipid levels profile in high-fat fed rats [6]. However, the constituents of persimmon leaves are resposible for producing these effects remain unclear. We recently demonstrated that standardized flavonoid extract from *Diospyros kaki* leaves (DK extract) exhibited the hypolipidemic effects using tyloxapol-injected mice, an animal model of endogenous dyslipidemia [7]. The effects of flavonoid extract from persimmon leaves in the exogenous dyslipidemic animals remain not adequately clear.

Diet-induced hyperlipemia is the most relevant stimulus for the induction of atherosclerotic lesions in humans. Thus, diet-induced hypercholesterolemia is almost always useful for the assessment of agents that interfere with absorption, degradation, and excretion of cholesterol, with minimal effects on cholesterol biosynthesis. Thus, elucidation of the anti-dyslipidemic effects of flavonoids extracted from persimmon leaves using diet-induced a

hypercholesterolemia model is needed. In the present study, we investigated the anti-dyslipidemic effects of the standardized flavonoid extract from *Diospyros kaki* L.f leaves (DK extract) in high-cholesterol diet (HCD) fed rats.

2. Materials and methods

2.1. Preparation of the DF extract

Diospyros kaki L.f leaves was collected from Lang Son province in June 2019. The plant identification was performed by Dr. Pham Thanh Huyen (Department of Medicinal Plant Resources, National Institute of Medicinal Materials (NIMM), Vietnam). The standardized flavonoid extract was prepared as previously report [7]. Briefly, 10 kg the leaves were cleaned, dried at 40-50°C and then cut into small pieces (2-5 mm). The dried leaves of persimmon were extracted three times with ethanol 70% (10/8/8 mL/g) at reflux for two hours, 1.5 hours and 1 hour, respectively. The collected extracts were pooled and evaporated the solvent to an extract of about 40% alcohol concentration. This EtOH extract was then adsorbed onto the column containing D101 macroporous resin. After finishing adsorption, washing with EtOH 30% until the color was faded. The column was eluted with EtOH 60%. The aqueous ethanol extract, after recovery of EtOH solvents by rotary evaporator, was evaporated by water bath at 60°C, and then dried under vacuum oven at 50°C until obtained 302 g dry extract (DK extract). The loss on drying of the DK extract was less than 5%.

The flavonoids content in DK extract was analyzed using HPLC method according to the previous described [8]. Briefly, the DK extract was hydrolyzed in a HCl 2M/methanol solution at 90°C for 2 hours. Quercetin and kaempferol content in hydrolyzed DK extract solution were determined by HPLC (Shimazu 20A) using Vertisep C_{18} column (250 mm × 4.6 mm, 5 µm) (Vertical Chromatography Co., Ltd, Bangkok, Thailand). The content of flavonoid in DK was calculated as in the following formular:

Total content of flavonoids in DK extract = (quercetin content × 2.55) + (kaempferol content × 2.59).

According to the analysis method, the total content of flavonoids in the DK extract was calculated as 21%.

2.2. Animals

Both male and female *Wistar* rats of 8-12 weeks old weighing between 180 and 220 grams were provided by Military Medical Academy, Hanoi, Vietnam. Rats were housed in the laboratory animal room (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*. Rats were kept for one week to acclimatize before the commencement of experiments.

2.3. Methods

High-cholesterol diet (HCD) was fed in rats by oral administration of oil-cholesterol mixture (cholesterol 10% (Merk, Germany), cholic acid 1% (Sigma, Singapore), propylthiouracil (Rieserstat®, Germany) 0.5%, and peanut oil added to precisely 1 mL) for four weeks according to the previous reported [9],[12].

Wistar rats were divided into 5 groups with 10 rats per group. Rats were given oral medication twice per day, at least two hours apart:

- Group 1 (Normal): distilled water 10 mL/kg b.w twice per day;
- Group 2 (HCD + vehicle): oil-cholesterol mixture 10 mL/kg b.w per day and distilled water 1 mL/100 g b.w per day;
- Group 3 (HCD + DK 50 mg/kg): oil-cholesterol mixture 10 mL/kg b.w per day and DK extract at the dose of 50 mg/kg b.w per day;
- Group 4 (HCD + DK 100 mg/kg): oil-cholesterol mixture 10 mL/kg b.w per day and DK extract at the dose of 100 mg/kg b.w per day
- Group 5 (HCD + atorvastatin 10 mg/kg): oil-cholesterol mixture 10 mL/kg b.w per day and atorvastatin (Stellapharm J.V. Co., Ltd.) with the dose of 10 mg/kg b.w per day.

At baseline and after 2 and 4 weeks of intervention, rats were fasted over night. Rat's body weight was evaluated. Blood was collected to measure serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C) concentrations and serum aminotransferases (alanine transaminase - ALT, aspartate aminotransferase - AST). Non-HDL-C concentration was calculated using the formula: non-HDL-C=TC−(HDL-C) (mmol/L) [10]. Biochemical analyzer (ERBA Chem, India) and commercial ERBA diagnostic kits were used for serum analysis of TC, TG, and serum aminotransferases.

2.4. Statistical analysis

SigmaPlot 12.0 (SYSTA Software Inc, Richmond, CA, USA) was used for statistical analysis. Values were presented as mean ± S.E.M. Data obtained were analyzed by a one-way analysis of variance (ANOVA) followed by post hoc Student-Newman-Keuls test for multiple comparisons. p values < 0.05 were considered statistically significant.

3. Results

Effects of DK extract on body weight of rats

As shown in Fig. 1, initial body weights of five groups were not significantly different (p>0.05). After 4 weeks of intervention, the body weight of HCD-treated vehicle rats was markedly reduced compared with the normal group (p<0.001). DK extract (50 and 100 mg/kg) and atorvastatin (10 mg/kg) significantly improved body weight loss compared with the model group animals at the end of experiment (p<0.05).

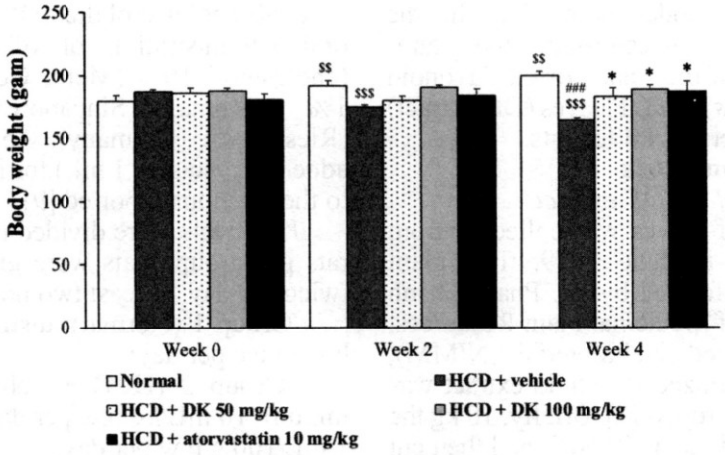

Fig. 1. Effects of DK extract on body weight of high-cholesterol diet fed rats.
Data were presented as the mean ± S.E.M. (n=10). *p<0.05 vs high-cholesterol diet fed rats-treated vehicle; ###p<0.001 vs normal rats; $$p<0.01, $$$p<0.001 vs Week 0 (ANOVA followed by post hoc Student-Newman-Keuls test).

Effects of DK extract on serum lipid profiles

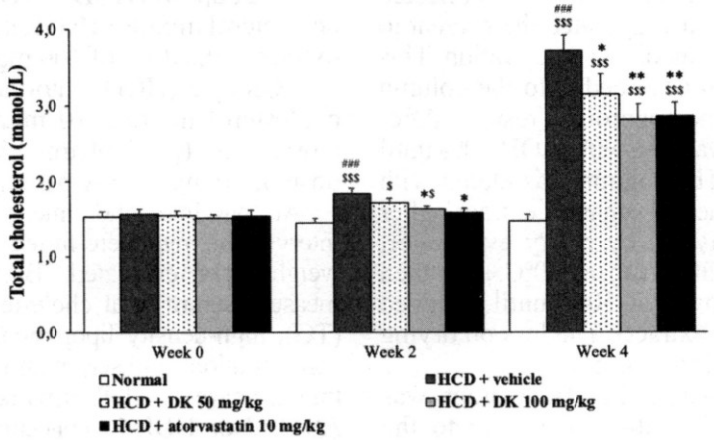

Fig. 2. Effects of DK extract on total cholesterol (TC) concentration in high-cholesterol diet fed rats.
Data were presented as the mean ± S.E.M. (n=10). *p<0.05, **p<0.01 vs vehicle-treated high-cholesterol diet fed rats; ###p<0.001 vs normal rats; $p<0.05, $$p<0.01, $$$p<0.001 vs Week 0 (ANOVA followed by post hoc Student-Newman-Keuls test).

After 2 and 4 weeks of intervention, there was a considerable increase in levels of TC as compared with the normal group (p<0.001). After 2 weeks of treatment, DK extract at the dose of 100 mg/kg and atorvastatin at dose of 10 mg/kg significantly reduced TC levels as compared with the model group (p<0.05). However, DK extract at the dose of 50 mg/kg insignificantly decreased levels of TC (p>0.05). At the end of experiment, DK extract at both doses and atorvastatin markedly lowered when compared to the model group (p<0.05) (Fig 2).

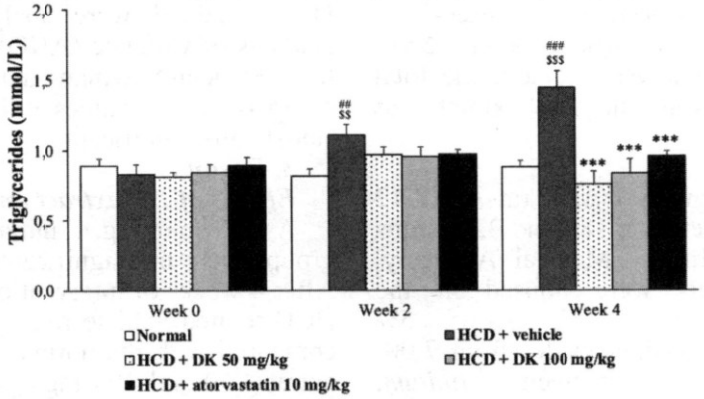

Fig. 3. Effects of DK extract on triglycerides (TG) concentration in high-cholesterol diet fed rats
Data were presented as the mean ± S.E.M. (n=10). ***p<0.001 vs vehicle-treated high-cholesterol diet fed rats; ##p<0.01, ###p<0.001 vs normal rats; $$p<0.01, $$$p<0.001 vs Week 0 (ANOVA followed by post hoc Student-Newman-Keuls test)

As shown in Fig. 3, HCD resulted in an obvious increase in triglycerides (TG) concentration in rats ($p<0.001$). After 2 week of treatment, no differential change was observed between groups treated with DK extract and atovastatin and the model group ($p > 0.05$). After 4 weeks of treatment, the groups treated with atorvastatin and DK extract markedly lowered TG as compared with the model group ($p<0.01$).

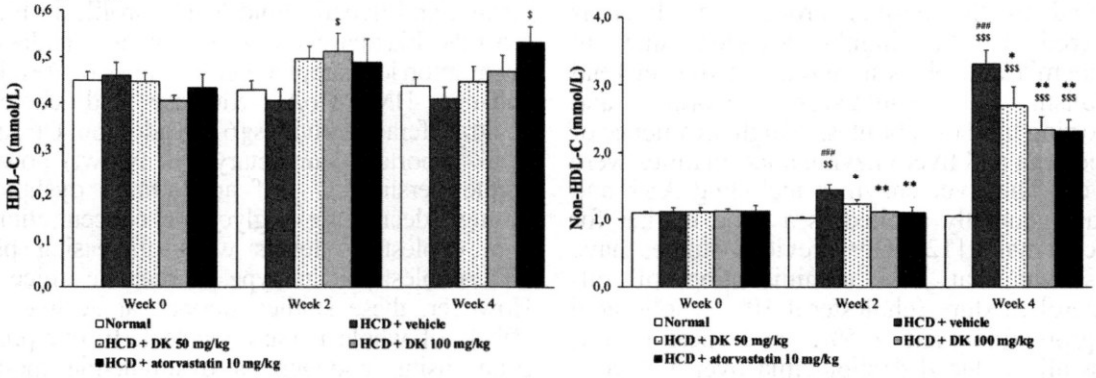

Fig. 4. Effects of DK extract on HDL-C and Non-HDL-C concentrations in high-cholesterol diet fed rats
Data were presented as the mean ± S.E.M. (n=10). *p<0.05, **p<0.01, ***p<0.001 vs vehicle-treated high-cholesterol diet fed rats; ###p<0.001 vs normal rats; $p<0.05, $$p<0.01, $$$p<0.001 vs Week 0 (ANOVA followed by post hoc Student-Newman-Keuls test).

Fig. 4 showed that there were no differences in the HDL-C levels in all groups when compared to the normal group ($p>0.05$). Level of non-HDL-C was increased comparing to the model group ($p<0.001$). That was apparent from the second week of intervention. The dyslipidemic animals treated with DK extract at both doses and atorvastatin observed a significant lowered non-HDL-C concentration compared to the HCD-treated vehicle group ($p<0.05$) after 2 and 4 weeks of intervention.

Effects of DK on serum aminotransferases

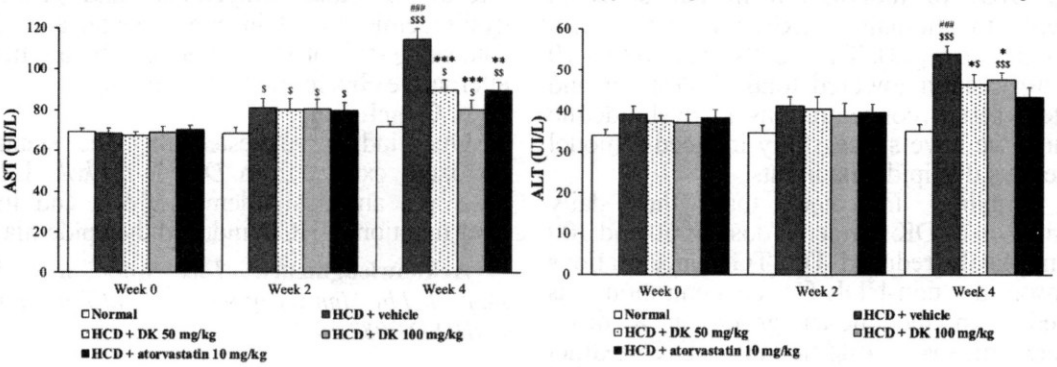

Fig. 5. Effects of DK on AST and ALT levels in high-cholesterol diet fed rats
Data were presented as the mean ± S.E.M. (n=10). *p<0.05, **p<0.01, ***p<0.001 vs vehicle-treated high-cholesterol diet fed rats; #p<0.05, ##p<0.01, ###p<0.001 vs normal rats; $p<0.05, $$p<0.01, $$$p<0.001 vs Week 0 (ANOVA followed by post hoc Student-Newman-Keuls test).

Since liver is the primary organ usually affected by hyperlipidemia, we performed the main liver function tests, such as serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities. As shown in fig. 5, serum ALT and AST levels in model rats were increased significantly when compared to the normal group after 4 weeks of intervention ($p<0.001$). Compared with the dyslipidemia model group the levels of AST and ALT reduced significantly in all DK extract and atorvastatin-treated groups after 4 weeks of treatment ($p<0.05$).

4. Discussion

In this study, HCD using oil-cholesterol mixture in 4 weeks period induced a significant development of dyslipidemia as indicated by increase TC, TG, HDL-C concentrations along with decrease of non-HDL-C concentration. In literature, the model of exogenous dyslipidemia in rats induced by oil-cholesterol mixture is widely used to evaluate the hypolipidemia effect of natural or synthetic drugs [9]. The addition of cholic acid and propylthiouracil aims to increase the absorption of cholesterol and reduce the conversion of cholesterol to bile acids, thus creating a dyslipidemic model with higher stability, homogeneity and shortening the study period. Furthermore, it is important to note that non-HDL-C represents the cholesterol content present in all the atherogenic lipoproteins. Moreover, non-HDL-C is easily calculated from a lipid profile. Therefore, non-HDL-C level was determined to

evaluate the anti-dyslipidemic effects of DK extract [11]. Besides blood lipid levels, liver enzymes are also indicators to evaluate the anti-dyslipidemic effects. In this study, elevation of serum AST and ALT enzymatic activity was observed in HCD rats compared to the normal group. The liver is considered to be highly affected due to dyslipidemia and plays a significant role in lipid metabolism by manufacturing, storing, and transporting lipid metabolites. A high prevalence of dyslipidemia and liver enzyme abnormalities were observed. The liver enzymes including AST and ALT are generally applied as a good marker for hepatic damage [12]. Our previous studies have also shown that oral administration of oil-cholesterol mixture (cholesterol 10%, cholic acid 1%, propylthiouracil 0.5%, and peanut oil) successfully induced dyslipidemia over a 4-week period [13].

We used atorvastatin, a selective, competitive inhibitor of HMG-CoA reductase, as a reference to examine the hypolipidemic abilities of DK extract in dyslipidemic rats. In clinical practice, atorvastatin is used as an adjunct to diet for reduction of elevated TC, LDL-C and triglycerides in adults. The usual dose of atorvastatin is 10 mg once a day. The maximum dose is 80 mg once a day [2],[14]. Dose of atorvastatin in our study is equivalent to human maximum dose with conversion ratio 6 [13]. The results showed that 10 mg/kg atorvastatin lowered total cholesterol and lipoprotein serum concentrations in dyslipidemic rats. Thus, we have successfully induced a model of exogenous dyslipidemia in rats.

Our finding indicated that the daily administration of DK extract at dose of 50 and 100 mg/kg markedly reduced TC, TG concentrations and lowered non-HDL-C concentration as compared with the model group in a dose-dependent manner. Additionally, DK extract significantly improved body weight loss and reduced serum aminotransferases enzymatic activity in HCD rats. According to the literature reviews, J. S. Lee et al. confirmed that the 5% (wt/wt) powdered whole persimmon leaf improves plasma and hepatic lipid levels profile in high-fat fed rats. Plasma cholesterol lowering mediated by persimmon leaf supplementation possibly resulted to enhance HMG-CoA reductase and cholesterol acyltransferase activities [6]. In addition, Un Ju Jung et al. reported that dietary 5% (wt/wt) powdered whole persimmon leaf ameliorated dyslipidemia through decreasing triglyceride concentration and total cholesterol levels while increasing plasma HDL-cholesterol in type 2 diabetic mice [15]. However, these studies stopped at evaluating the effects of whole persimmon leaf. In our previous study using endogenous dyslipidemia model in mice, we demonstrated that DK extract at dose 100 and 300 mg/kg exhibited the hypolipidemic effect throught decrease the levels of TG [7]. In the current study, the effects of treatment of standardized flavonoid extract from persimmon leaves in exogenous dyslipidemic animals model more comprehensively evaluated. The results of this study strongly confirm the anti-hyperlipidemic effect of persimmon leaves and the role of flavonoids in these effects in the exogenous and endogenous dyslipidemia model in experimental animals. Our data suggests that DK extract can be applied as an alternative choice for dyslipidemia.

5. Conclusion

Our finding suggested that the standardized flavonoid extract from *Diospyros kaki* L.f leaves possesses anti-dyslipidemic effects and improves liver function in HCD-induced dyslipidemia in rats.

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References

1. Rader D. J., Hobbs H. H. (2005), *Disorders of lipoprotein metabolism*, 16th ed. Harrisons Principles of Internal Medicine, 2286. **2**. ACC/AHA (2019), Cholesterol Management, *ACC/AHA Updates Guideline*, 99(9), 589-591. **3.** Golomb B. A., Evans M. A. (2008), Statin adverse effects, *American Journal of Cardiovascular drugs*, 8(6), 373–418. **4.** Che C. T., Wang Z., Chow M., Lam C. (2013), Herb-herb combination for therapeutic enhancement and advancement: theory, practice and future perspectives, *Molecules*, 18(5), 5125–5141. **5.** Xie C., Xie Z., Xu X., Yang D. (2015), Persimmon (*Diospyros kaki* L.) leaves: a review on traditional uses, phytochemistry and pharmacological properties, *Journal of Ethnopharmacology*, 163, 229–240. **6**. Lee J. S., Lee M. K. Ha T. Y., Bok S. H., Park H. M., Jeong K. S., Woo M.N., Do G. M., Yeo J. Y., Choi M. S. (2006), Supplementation of whole persimmon leaf improves lipid profiles and suppresses body weight gain in rats fed high-fat diet, *Food and Chemical Toxicology*, 44, 1875–1883. **7**. Nguyen Thi Thanh Loan, Le Thi Xoan, Nguyen Thi Phuong, Nguyen Van Tai, Nguyen Thi Bich Thu (2020), Modulation of dyslipidemia on tyloxapol-induced hyperlipidemia in mice by flavonoid-enriched extract from *Diospyros kak*i L.f leaves, *Journal of Medicinal Materials*, 25(4), 246-250. **8**. Le Thi Xoan, Dang Thi Hanh, Tran Nguyen Hong, Nguyen Thi Phuong, Nguyen Van Tai, Pham Thi Nguyet Hang, Ha Van Oanh (2018), Neuroprotective effects of flavonoid-enriched extract from *Diospyros kaki* leaves on cerebral ischemia-induced injury in mice, *Journal of Medicinal Materials*, 23(2), 104-110. **9.** Nassiri-Asl M., Zamansoltani F., Abbasi E., Daneshi M. M., Zangivand A. A. (2009), Effects of *Urtica dioica* extract on lipid profile in hypercholesterolemic rats, *Journal of Chinese Integrative Medicine,* 7(5), 428–433. **10**. Sanchez-Muniz F. J., Bastida S. (2008), Do not use the Friedewald formula to calculate LDL-cholesterol in hypercholesterolaemia rats, *European Journal of Lipid Science and Technology*, 110, 295–301. **11**. Virani S. S. (2011), Non-HDL Cholesterol as a Metric of Good Quality of Care, Texas Heart Institute, 38(2), 160–162. **12**. Kathak R. R., Sumon A. H., Molla N. H., Hasan M., Miah R., Tuba H. R., Habib A., Ali N. (2022), The association between elevated lipid profile and liver enzymes: a study on Bangladeshi adults, *Scientific Reports*, 12 (1711), 1-8. **13**. Pham Ba Tuyen, Truong Thi Huyen, Dinh Thi Thu Hang, Pham Thi Van Anh (2021), A Novel Herbal Medicine for Dyslipidemia: Assessments in Experimental Models, *Evidence-Based Complementary and Alternative Medicine*, 5529744, 1-5. **14**. Ciffone N. A., Copple T. (2019), Managing dyslipidemia for CVD prevention. *The Nurse Practitioner*, 44(1), 8–16. **15**. Jung U. J., Park Y. B., Kim S. R., Choi M/. S. (2012), Supplementation of Persimmon Leaf Ameliorates Hyperglycemia, Dyslipidemia and Hepatic Fat Accumulation in Type 2 Diabetic Mice, *PLoS One*, 7(11), e49030, 1-11.