Assessment of anti-atherosclerotic effect of *Eurycoma longifolia* extract on high-fat diet model in rats. I: Histological study

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SUMMARY

The objective of this study was to examine the effect of *Eurycoma longifolia* (EL) extract on intima media thickness (IMT) of aorta in Sprague Dawley (SD) rats fed with high-fat diet. Twenty healthy male SD rats were divided into 4 groups of 5 animals each and treated for 12 weeks as follows: Group ND was given only normal diet, Group NDEL was given normal diet and EL, Group HFD was given only high fat diet, Group HFDEL was given HFD and EL extracts. The aortic thicknesses of the intima media were photographed and measured with Dino-Capture® 2.0.In this study, the HFD produced obvious atherosclerotic plaque; treatment with aqueous extract of EL has reduced the size and formation of atherosclerotic plaque. The aortic IMT was more significantly increased in the HFD group than that of ND ($p < 0.05$); on the other hand, the aortic IMT was more significantly reduced in HFDEL group than that of HFD group ($p < 0.05$). It is concluded that the aqueous extract of EL significantly attenuated the formation of atherosclerotic plaques in the aorta of rats and preserved the vascular structure.

Key words: Atherosclerosis – Intima media thickness – Aorta – High fat diet – Rats – Tongkat ali

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INTRODUCTION

In the last two decades, there has been an increase in the prevalence of chronic diseases such as heart disease, hypertension and atherosclerosis. These diseases are currently the major causes of morbidity and mortality around the world (Kones, 2011). Many researchers have defined atherosclerosis as a slow, progressive, and chronic inflammatory disease (Toth, 2008; Galkina and Ley, 2009). It has multistep processes leading eventually to morphological changes of cells and receptors of both the blood vessels and endothelial cells (Rajendran et al., 2013). It is predominantly dependent on potential risk factors such as hyperlipidemia, hypertension and male gender (Cizek et al., 2007).

The atherosclerotic lesion mainly targets the large and medium-sized muscular arteries (Merino et al., 2014). In general, atherosclerosis could be defined as a condition of chronic and progressive inflammatory response which is characterized by endothelial dysfunction (ED). The hallmarks of ED are macrophage-foam cell formation, production of inflammatory cytokines and over-expression of adherent molecules such as vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (Merino et al., 2014). The role of oxidative-modified LDL (ox-LDL) is very clear nowadays. Ox-LDL is mainly taken up by macrophages using scavenger receptors; eventually the macrophages turn into foam cells (Truman et al., 2014). Foam cells secrete

cytokines and amplify the production of free radicals, which includes the reactive oxygen species (ROS) (Jaffer et al., 2006; Hajjar et al., 2009). Consequently, the increased production of ROS may reduce the production and bioavailability of nitric oxide (NO) in endothelial cells which mediate cell damage and lead to vasoconstriction (Elahi et al., 2009). All these disorders exacerbate the situation in vulnerable arteries, and induce a series of inflammatory reactions and oxidative stress (Wittchen, 2009).

Intima media thickness (IMT) is considered a primary marker for CV risk at an early stage of atherosclerosis. IMT is approximately equal to the total thickness of the tunica intima and tunica media (Onut et al., 2012). *Eurycoma longifolia* (EL) is an evergreen shrub commonly found in tropical regions of Asia; it has been used since ancient times to combat many health problems and diseases. In Malaysia, it is commonly called Tongkat ali and has been traditionally used for its antimalarial, aphrodisiac, antidiabetic, antimicrobial, and antipyretic activities, as well as for improvements in physical and mental energy levels and overall quality of life (Bhat and Karim, 2010). Evidence indicates that EL can exert several health-beneficial effects and improve general well-being (Talbott et al., 2013). Phytochemical studies on this plant revealed that EL contains valuable bioactive compounds such as quassinoids (Tran et al., 2014). However, there is little information concerning the ability of EL to inhibit the progression of atherosclerosis at the level of vasculature system. To the authors' knowledge, there are no published data on the effects of EL on cardiovascular diseases (CVDs), and atherosclerosis in particular. Thus the objective of the present study was to investigate the effect of oral administration of EL on atherosclerotic lesion formation in rats fed high-fat diet (HF).

MATERIALS AND METHODS

Eurycoma longifolia extract

The EL extract powder PHYSTA®, was obtained from Biotropics Malaysia Berhad. The aqueous extract was prepared by dissolving 15 mg of EL in 10 ml of distilled water, and this allowed simple and easy weekly dose calculations in accordance with the rat's body weight. The prepared solution was kept in a refrigerator at temperature of 2-8ºC, and was removed from the refrigerator 30 minutes before its administration so as to allow the equilibrium to reach to the rat's body temperature.

High fat diet

HFD Pellets were purchased from (MP Biomedicals, California, USA, Next Gene Scientific Sdn. Bhd). The composition of high saturated fat diet was as shown in Table 1.

Animals and experimental design

Twenty young, adult male Sprague Dawley (SD) rats weighing 250-300 g were housed in standard plastic cages (2 rats per cage). They were maintained at room temperature (22–24°C) with adequate ventilation, 12-h light-dark cycle and about (50±5%) humidity. After one week of acclimatization, they were randomly divided into four groups of 5 animals each and treated for 12 weeks as follow: Group ND was given only normal diet, group NDEL was given normal diet and EL extracts (15 mg/kg) dissolved in distilled water, group HFD was given only high fat diet and group HFDEL was given high fat diet and EL extracts (15 mg/kg). The EL extract was administered by gastric gavage. The animals were treated according to the Standards and Regulations for the Care and Use of Laboratory Animals of the National Institutes of the Health and according to the guidelines of IIUM animal Ethical Committee, the reference number (IIUM/519/14/4/IACUC).

Tissue specimens

At the end of 12th week, the rats were kept in fasted state for 12 hours prior to anesthesia and then sacrificed by cervical dislocation. The aortic arteries were cleaned of loose connective tissue and the adherent tissues, rinsed with normal saline; care was taken to avoid any damage to the tissue. The specimens were fixed in 10% formal saline, underwent a dehydration process with gradual series of alcohol, embedded in paraffin

Table 1. The composition of high fat diet

INGREDIENT	AMOUNT
Casein Purified High Nitrogen	4000 gm.
DL-Methionine	60 gm.
Sucrose	6116 gm.
Corn Starch	4000 gm.
Coconut Oil Hydrogenated	4000 gm.
Alphacel, Non-Nutritive Bulk	1000 gm.
DL-a-Tocopherol Powder (250 IU/gm.)	24 gm.
AIN-76 Mineral Mix	800 gm.

Plus MP Vitamin diet fortification mixture 1.2 x normal

Table 2. Intima media thickness (IMT) of different groups at the end of the study

Groups	Intima media thickness (mm)
ND	0.082± 0.005
NDEL	0.082± 0.005
HFD	0.246± 0.027*
HFDEL	0.102±0.013#

Values are presented as means ± SD. *Significantly (P<0.05) different from ND. #Significantly (P<0.05) different from HFD.
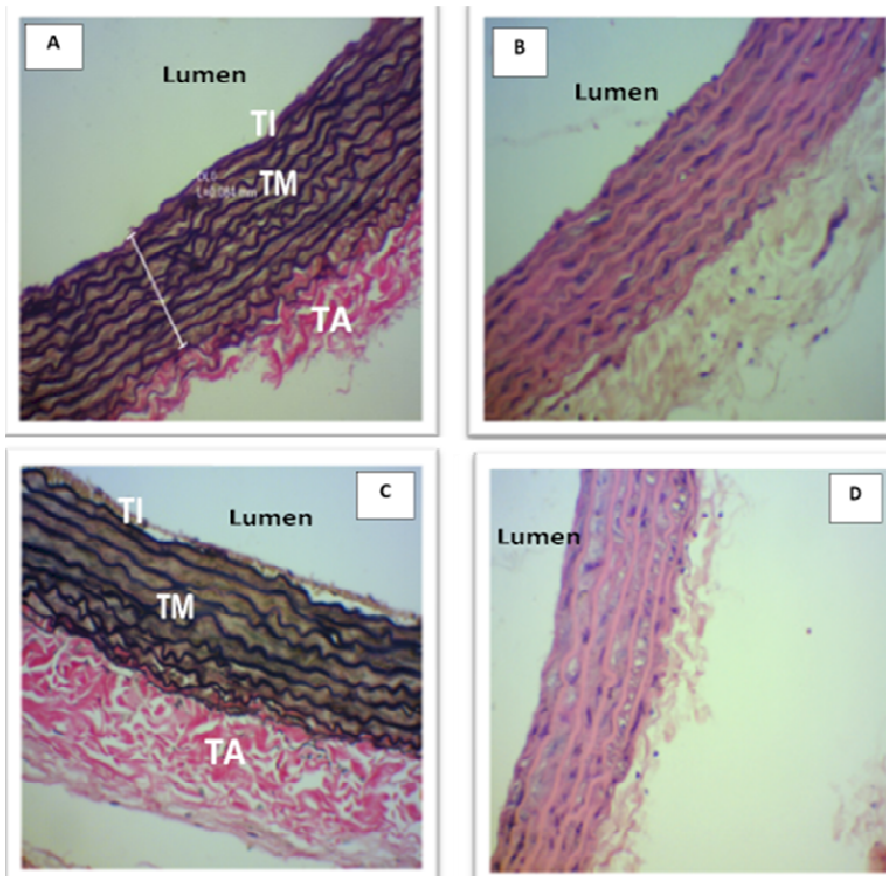
Fig. 1. Transverse section of aorta of ND (A&B) and NDEL (C&D) groups. Notice that there are no differences between the two groups. (TI; Tunica intima, TM; Tunica media, TA; Tunica adventitia, I; Intima media thickness). A&C are stained by VVG. B&D are stained by H&E. Original magnification x20.

wax and sectioned by rotary microtome at 5 μm and stained with hematoxylin and eosin (H&E) and Verhoeff–Van Gieson (VVG). Finally, the specimens were analyzed via microscope and Dino-Capture 2.0. The histological examination was carried out at a magnification of ×20 with a light microscope. The thicknesses of the intima media of the aortas were photographed and measured with Dino-Capture® 2.0. The distance between the lumen and the internal elastic lamina was measured as intima media thickness (IMT), and the internal elastic lamina was identified by VVG staining. Atherosclerosis was examined in a blinded manner using five transverse sections from each group.

Statistical analyses

The statistical package for the social sciences (SPSS) software (version 22) was used to analyze the data. Original data were expressed as means (standard deviations). Homogeneity of variances was evaluated with the Levene test. One-way analysis of variance (ANOVA) was performed. When the P values were statistically significant, a post-hoc Tukey honestly significant difference was used to determine which groups differed from the others. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Effects of EL administration and high fat diet on IMT

IMT values are given in Table 2. Compared to ND group, NDEL and HFDEL did not exhibit a statistical significance in IMT values which were (0.082 ± 0.005) and 0.102(0.013), respectively, ($P>0.05$), while HFD had a higher value of IMT (0.246(0.027)) than ND (0.082(0.005)), which was statistically significant ($P<0.05$). Moreover, the oral administration of EL (15 mg/kg/day) attenuated the formation of atherosclerotic plaques in HFDEL and did not affect the integrity and the normal architecture of aorta in NDEL. Furthermore, the morphological measurements of IMT of HFDEL showed a significant improvement (0.102(0.013)) compared to HFD (0.246(0.027)), ($P<0.05$) (Table 2).

Histopathological changes of aorta

Light microscopic examination of the aorta of the control rats (ND group) showed normal structural features of the aorta revealing normal components of the TI and TM (Fig. 1A and B). Treatment of rats with EL (NDEL group) did not exhibit any morphological changes in compression with ND control group; moreover, there was no atherosclerotic lesion at all, (Fig. 1C and D). On the other hand, the aorta in the HFD-fed rats displayed thickness in the tunica intima and tunica media, and there was a large deposition of lipid in the tunica intima of the aorta in HFD compared with ND group. Furthermore, VVG staining showed a reduction in the

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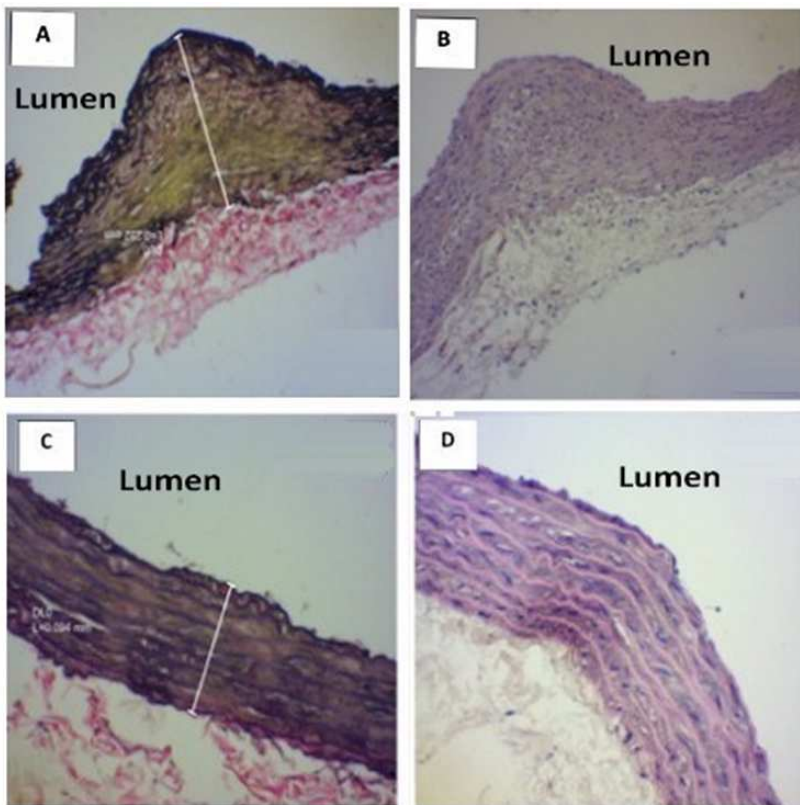
Fig. 2. Transverse section of aorta of HFD (A&B) and HFDEL (C&D) groups. Notice the atherosclerotic lesion and lipid deposits in the tunica media (Yellow arrow) in aorta of HFD group characterized by its thickness relative to the ND group; treatment with EL (HFDEL group) attenuated the pathological lesion observed in the HFD group. (I; Intima media thickness). A&C are stained by VVG. B&D are stained by H&E. Original magnification x20.

elastic fibers in the tunicae of the aorta in the HFD group (Fig. 2A). These alterations were reduced with the administration of EL extract for twelve weeks in HFDEL group, and showed significant improvement in the histological changes (Fig. 2C and D). In addition, there were no marked histological changes and significant improvements in the deposition of elastic fibers were observed in HFDEL as evident by VVG staining.

DISCUSSION

The present study has demonstrated that treatment of rats with EL aqueous extract exhibited a marked beneficial effect on atheromatus plaques. Atherosclerosis can be considered a significant cause of mortality and morbidity worldwide (Upadhyay, 2015). HFD animal model had been well established to study the effect of various preparations/drugs on atherosclerosis formation. HFD animal model has been used for decades and significantly contributed to the analysis of the pathophysiology of atherosclerosis lesion in rats (Wu et al., 2014). For extrapolating the effect of EL extract to humans, the HFD atherosclerotic model was the most relevant animal model for this study, based on many studies which confirmed that the murine models are the relevant animal model, as well as the most extensively used to develop atherosclerosis. In the absence of standardization of the composition of HFD used and exposure time to the diet in those stud-ies, we have chosen a specified 53% HFD and a period of 12-week study, as the atherosclerosis requires certain serum cholesterol levels over certain periods of time. Although the evidence about time frame is slim, the majority of researchers have been using a time frame from six to fourteen week studies (Elmarakby and Imig, 2010). Experimentally, it has been suggested by some researchers that rats fed an HFD maintain metabolic homeostasis for approximately six weeks, and then they start to develop hyperlipidemia, hepatic-steatosis (i.e. fatty liver) and metabolic syndrome (Kim et al., 2012).

Thus, the histological findings of the present study showed that the HFD atherosclerotic model is indeed a feasible and efficient model to study atherosclerotic lesion. It is interesting and surprising that the administration of EL aqueous extract resulted in a drastic suppression of the atherosclerosis lesion in HFDEL compared with HFD. To our knowledge, this is the first report of IMT values among rats treated with EL extract. Generally, increased IMT of the aorta is a reflection of atherosclerotic burden and a predictor of subsequent cardiovascular events. Given the normal histologic architecture of the aorta in NDEL and HFDEL, it can be speculated that EL extract might has anti-atherogenic property, genes experimental study has confirmed the present histological observation (the authors, unpublished data); however, further molecular studies are needed to investigate the mechanisms of this hypothesis.

It has been suggested that the LDL particles are more susceptible to pass and go behind the endothelium layer because it has a small density. Once the LDL penetrates the arterial wall of blood vessels, the LDL particles and their content are prone to oxidize. Macrophages ingest oxidized LDL particles, forming specialized foam cells. The foam cell is not able to process the oxidized LDL and eventually ruptures, leaving behind oxidized materials and fats in the artery wall. This attracts more monocytes and macrophages, and triggers a cascade of immune responses which over time can produce an atheroma (Soloperto and Casciaro, 2012). It becomes very obvious that the earliest vascular change described microscopically is the intimal thickening. During the atherosclerotic development, the endothelium, innermost layer, becomes the primary target that has been attacked, causing endothelial cell proliferation and smooth muscle cells (SMCs) migration, which lead to vascular endothelial cells' permeability to a certain lipoprotein such as oxidative-modified LDL (Adam et al., 2009). In this investigation, the EL extract was observed to be effective in counteracting the atherogenic effect of HFD on intima media thickness. It is likely noticeable that the HFD group showed some pathological abnormalities in the aorta, both endothelial and subendothelium layers and smooth muscle parts are affected with consuming HFD, compared to the normal morphology of ND. These data combined with the possibility of HFD to reduce the hemodynamic shear stress and change the conformational shape of LDL receptors, eventually increasing the cholesterol level. Hyper-cholesterolemia enhances the adhesion of leukocyte to endothelium (Zhang et al., 2011). The effect of EL on aorta morphology demonstrated some positive protective findings. There were remarkable improvements observed in the HFDEL group compared to the severe deterioration in aorta layers of HFD group, indicating the vascular protective potential of EL. These deleterious effects of HFD have been abrogated with the administration of EL extract for twelve weeks. This suggests that EL extract exhibits anti-atherogenic potential possibly via an anti-hyperlipidemic property (i.e., vascular protective potential). The anti-atherogenic effect of EL might be due to the limitation of LDL permeability throughout the endothelial cells, suppression SMCs proliferation and migration by modulating the endothelium layers. Actually, the adhesion between macrophages and vascular endothelial cells is the main initiator of atherosclerosis (Yuan et al., 2012). This mechanism was avoided by down-regulation of vascular cell adhesion molecule-1 (VCAM-1) expression in the aorta (Smedlund and Vazquez, 2008).

Administration of EL extract maintained the integrity of elastic fibers in tunica media in atherosclerotic treated rats (HFDEL) group. Thus, the present results clearly indicate that the EL extract has a promising ameliorative effect on the atheromatus plaque in the HFD model rat.

In conclusion, the present study demonstrates that the EL extract significantly prevents the progression of atherosclerosis in the HFD model of rats and preserves the vascular structure. Therefore, the *Eurycoma longifolia* extract may prove to be a potential therapy for atherosclerosis in human beings.

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