UNIVERSITI PUTRA MALAYSIA

PROPHYLACTIC AND THERAPEUTIC EFFICACIES OF DIETARY ZERUMBONE SUPPLEMENTATION ON THE PATHOGENESIS OF ATHEROSCLEROSIS IN CHOLESTEROL-FED RABBITS

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By

HEMN HASSAN OTMAN HASSAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

This Dissertation is Dedicated to

My Beloved Wife

My Wonderful Triplets

My Lovely Parents and Siblings

All My Sincere Teachers, Lecturers and Friends
PROPHYLACTIC AND THERAPEUTIC EFFICACIES OF DIETARY ZERUMBONE SUPPLEMENTATION ON THE PATHOGESIS OF ATHEROSCLEROSIS IN CHOLESTEROL-FED RABBITS

By

HEMN HASSAN OTHMAN HASSAN

November 2014

Chairman: Professor Noordin Mohamed Mustapha, PhD

Faculty: Veterinary Medicine

Owing to the high incidence of cholesterol-induced cardiovascular disease particularly atherosclerosis. Furthermore, difficulty in finding a relatively efficacious, non toxic, readily available and cheap naturally existing antihyperlipidaemic and antiatherogenic complementary medicine warrant the search for such an agent. On the other hand, concerning the dangerous side effects of the chemical remedies utilized as a lipid lowering agents, therefore the current study was designed to investigate the prophylactic and therapeutic efficacies of dietary zerumbone (ZER) supplementation on the formation and development of atherosclerosis in rabbits fed with high cholesterol diet. A total of 72 New Zealand white rabbits (NZW) were divided randomly on two experimental studies carried out eight weeks apart.

First experiment was designed to investigate the prophylactic efficacy of ZER in preventing early developed atheromas lesion. A total of 30 healthy rabbits were equally allotted in to five groups comprising of six animals each, namely control (CN), hypercholesterolemic diet (HCD) and ZER preventive groups (ZPI, ZPII and ZPIII). The second experimental trial is aimed at investigating the therapeutic effect of ZER in reducing the atherosclerotic lesion progression and establishment, wherein 42 healthy NZW rabbits were equally assigned to seven groups comprising of six animals each, namely control (CN), high-cholesterol diet (HCD), ZER treatment groups (ZI, ZII and ZIII), Simvastatin (SIM) group (SG) and zerumbone-simvastatin (ZER-SIM) combination group (ZSG). Rabbits in CN group were fed a standard rabbit chow, whereas those in the HCD, ZPI, ZPII, ZPIII, ZI, ZII, ZIII, SG and ZSG given a cholesterol-rich diet (1%) (1g cholesterol/100g pellet). However, rabbits in the ZPI, ZPII and ZPIII preventive groups given ZER at a dose of 8, 16 and 20 mg/kg respectively, two weeks prior to the onset of lipedemia induction and then with the course of cholesterol-rich diet as a prophylactic measure. On the other hand, those in the ZI, ZII and ZIII treatment groups were given ZER at a dose of 8, 16 and 20 mg/kg
respectively, together with SIM at a dose of 15 and 5 mg/kg/day in SG and ZSG groups, respectively as a therapeutic measure.

Rabbits were sacrificed and thoracic aortas simultaneously with the vital internal organs were collected at 10 weeks post cholesterol-feeding for the prophylactic trial and 14 weeks concerning the therapeutic trial. In regard to the second trial, rabbits received treatments for about four weeks after cessation of cholesterol-rich diet at 10th weeks.

Following four weeks of supplementary treatment subsequent to high-cholesterol diet cessation at 10th week of 2nd experiment, ZER significantly reduce the serum lipid profile in all treated groups in a dose dependant manner as compared to non treated hypercholesterolemic animals. Sudanophilia, histopathological and ultrastructural changes show pronounced reduction in the plaque size in ZER medicated aortas. On the other hand, dietary supplementation of ZER for almost 10 weeks as a prophylactic measure indicates substantially decreasing in the lipid profile values and similarly plaque size in comparison with high-cholesterol non-supplemented rabbits.

Furthermore, the results of oxidative stress and antioxidant biomarkers evaluation indicate that ZER is a potent antioxidant in suppression the generation of free radicals in term of atherosclerosis prevention and treatment. ZER significantly reduces the value of MDA and augments the value of SOD. Zerumbone significantly reduces the incidence of inflammatory response in the process of atherosclerosis formation and development through significant suppression of proinflammatory mediators NF-κB, iNOS and COX-2, in turn reduce the inflammatory cytokines secretion TNF-α, IL-6, IL-1, and IF-γ evaluated by Western blotting and enzyme immunoassay techniques respectively.

Conversely, reduction and suppression of inflammatory mediators will contribute to minimizing the chronic inflammatory cells mainly macrophages recruitment to the lesion and foam cell formation which is evident by immunohistochemistry and fluorescent assay of RAM-11. ZER significantly reduces the expression of RAM-11 in the intimal plaque in all ZER supplemented groups in a dose dependent manner.

Moreover, ZER significantly reduces the proliferation and migration of vascular smooth muscle fibers through immunohistochemical and fluorescent detection of HHF-35 toward the intimal layer via induction of apoptosis, which is evident by down regulation of Bcl-2 and up regulation of Bax significantly evaluated by Western blotting technique. Furthermore, in vivo antiproliferative assay of ZER determined morphologically by TUNEL assay.

In conclusion our data indicate that, dietary supplementation of ZER at doses of 8, 16 and 20 mg/kg alone as a prophylactic measure and as a supplementary treatment with simvastatin, significantly reduces early plaque formation, development, and
establishment via significant reduction in serum lipid profile together with suppression of oxidative damage, therefore alleviate atherosclerosis lesions. Simultaneously, ZER significantly suppresses inflammatory reaction within the plaque consequently prevent foam cell formation and plaque progression. Finally, ZER significantly lessens smooth muscle cells proliferation via induction of apoptosis eventually reduce the plaque size.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KEBERKESANAN DIET ZERUMBON SEBAGAI PROFILAKSIS DAN TERAPEUTIK PERKEMBANGAN ATEROSKLEROSIS PADA ARNAB

Oleh

HEMN HASSAN OTHMAN HASSAN

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Berasaskan kepada insidens penyakit kardiovaskular teraruh-kolesterol terutamanya arteriosklerosis, satu kajian telah dibentuk untuk menyiasat kemujaraban profilaksis dan terapeutik pemberian zerumbon (ZER) rangsum terhadap pembentukan dan perkembangan arteriosklerosis pada arnab yang diberi rangsum tinggi kolesterol. Sebanyak 72 ekor arnab NZW dibahagikan secara rawak kepada dua kajian berjarak lapan minggu antaranya. Ujikaji pertama dibentuk untuk menyiasat kesan profilaksis kemujarahan ZER dalam mencegah pembentukan lesi awal arteroma. Sebanyak 30 ekor arnab sihat dibahagikan secara rata kepada lima kumpulan yang setiapnya mengandungi enam ekor iaitu, kawalan (CN), rangsum hiperkolesterol (HCD) dan kumpulan pencegahan ZER (ZPI, ZPII dan ZPIII).

Ujikaji kedua disasar untuk menyiasat kesan ZER dalam mengurang kemajuan serta pengukuhan lesi arteriosklerosis oleh ZER, dimana sebanyak 42 ekor arnab sihat NZW dibahagian secara rata kepada tujuh kumpulan mengandungi enam ekor arnab setiap satu, iaitu kawalan (CN), rangsum kolesterol tinggi (HCD), rawatan ZER (ZI, ZII dan ZIII), SIM (SG) dan gabungan ZER-SIM (ZSG). Arnab dalam kumpulan CN diberi makan makanan piawai arnab manakala dalam kumpulan HCD, ZPI, ZPII, ZPIII, ZI, ZII, ZIII, SG dan ZSG masing-masing diberi rangsum tinggi kolesterol (1%) (1g kolesterol/100 g makanan). Bagaimanapun, arnab dalam kumpulan pencegahan ZPI, ZPII dan ZPIII masing-masing menerima ZER pada dos 8, 16 dan 20 mg/Kg, dua minggu sebelum kejadian aruhan lipidemia dan kemudiannya dengan rawatan rangsum tinggi kolesterol sebagai langkah pencegahan.

Sebaliknya, kumpulan rawatan ZI, ZII dan ZIII menerima ZER masing-masing pada dos 8, 16 dan 20 mg/Kg bersamaan dengan SIM pada dos 15 dan 5 mg/kg/day pada kumpulan SG dan ZSG sebagai langkah terapeutik. Selepas dikorbankan, aorta toraks
bersamaan dengan organ dalam penting lain diambil pada minggu ke 10 pasca pemberian kolesterol untuk kajian profilaksis. Dalam kajian terapeutik, arnab menerima rawatan selama empat minggu selepas 10 minggu pemberian rangsum kolesterol. Sepanjang empat minggu rawatan tambahan selepas pemberhentian rangsum tinggi kolesterol pada minggu ke-10 di ujikaji kedua, ZER telah mengurang profil lipid serum secara keertian pada semua kumpulan rawatan dalam pola bergantung dos berbanding dengan kumpulan kolesterol tinggi tak terawat.

Sudanofilia, perubahan histopatologi and ultrastruktur menunjuk pengurangan ketara saiz plak pada aorta haiwan terawat ZER.

Sebaliknya, rawatan ZER selama hampir 10 minggu sebagai langkah profilaksis menandakan pengurangan ketara nilai profil lipid dan saiz plak berbanding dengan kumpulan tak terawat. Tambahan lagi, keputusan tegasan oksidatif dan biopenanda anti-pengoksidaan menunjukkan kemanjuran ZER dalam menindas penjanaan radikal bebas berasaskan pencegahan dan rawatan arteriosklerosis. ZER juga secara keertian telah mengurang nilai MDA dan mengganda nilai SOD. Zerumbon telah secara keertian mengurang insidens gerakbalas inflamasi dalam proses pembentukan dan perkembangan ateriosklerosis melalui penindasan keertian perantara proinflamasi seperti NF-κB, iNOS dan COX-2 yang seterusnya mengurang rembesan sitokin inflamasi seperti TNF-α, IL-6, IL-1, dan IF-γ yang dinilai melalui teknik asai penyerapan Western.

Pengurangan dan penindasan perantara inflamasi akan menyumbang kepada pengurangan sel inflamasi kronik terutamanya kemasukan makrofaj ke lesi dan pembetukan sel busa yang dibuktikan dengan imunohistokimia dan asai pendaflour RAM-11. ZER secara keertian telah mengurang penjelmaan RAM-11 pada plak intima di kesemua kumpulan pemberian tambahan ZER dengan pola bergantung dos. Tambahan lagi, ZER secara keertian mengurang pemproliferatan dan penghijrahani otot licin vaskular melalui penjelmaan imunohistokimia dan pendaflour HHF-35 terhadap lapisan intima melalui paruan apoptosis yang terbukti dengan penurunawalatur Bcl-2 dan tingkatkuawalatur Bax secara keertian yang dinilai dengan teknik penyerapan Western. Tambahan lagi, anti-pemproliferatan in vivo oleh ZER yang dibuat secara morphologi dengan asai TUNEL.

Sebagai rumusan, data yang terhasil menunjukkan bahawa pemberian makan ZER sahaja pada dos 8, 16 dan 20 mg/Kg untuk langkah profilaksis dan tambahan rawatan dengan SIM, mengurang pembetukan, perkembangan dan penghijrahani awal plak secara keertian melalui penurunan profil lipid berserta penindasan kerosakan oksidatif yang mengurang lesi arteriosklerosis. Malah gabungan ZER menindas secara keertian tindakbalas inflamasi dalam plak yang akhirnya menghalang pembnetukan sel busa dan perkembangan plak. Akhir sekali, ZER secara keertian mengurang pemproliferatan otot licin melalui aruhan apoptosis yang mengecil saiz plak.
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I certify that a Thesis Examination Committee has met on 12 November 2014 to conduct the final examination of Hemn Hassan Othman on his thesis entitled "Prophylactic and Therapeutic Efficacies of Dietary Zerumbone Supplementation on the Pathogenesis of Atherosclerosis in Cholesterol-Fed Rabbits" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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# 5 ANTI-INFLAMMATORY AND ANTI-PROLIFERATIVE EFFICACIES OF ZERUMBONE ON THE INITIATION, PROPAGATION AND ESTABLISHMENT OF ATHEROSCLEROSIS IN HYPERCHOLESTEROLAEMIC RABBITS

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4.29 Bar graphs represent hypolipidemic effect of ZER on LDL-C (C) and HDL-C (D). SG and ZSG groups display significant P<0.05 reductions in LDL-C. Similarly, HDL-C shows reduction SG and ZSG groups, however the reduced values are not significant as LDL-C.

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4.33 Photomicrograph of aorta of CN and HCD groups. Aorta in HCD group demonstrates nearly occupied thick fibro-muscular plaque (P), consist of many lipid-laden foam cells at the base (yellow arrow), highly proliferated VSMCs and fibroblasts toward the intima (black arrow), pinkish ground substances (dashed black arrow), and many lymphocytes at the top of the plaque (dashed yellow arrow). Aorta in CN group shows no lesion. L: Lumen; I: Intima; M: Media; A: Adventitia. H&E. Scale bars: 500 µm.

4.34 Photomicrograph of aortas from HCD, ZI, ZII and ZIII groups. Aortas in ZI, II and III show significant reduction in the plaque (P) thickness and foam cells (black arrows) compare to the thick fibromuscular plaque of HCD group. Yellow arrow in ZI group refers to the proliferative VSMCs. L: Lumen; M: Media; A: Adventitia. H&E. Scale bars: 500 µm.

4.35 Photomicrograph of aorta from SG and ZSG groups. Aortas in both SG and ZSG groups display significant reduction in foam cells (black arrow) and increase in pinkish ground substances (dashed black arrow). Plaque reduction is much prominent in ZSG group. L: Lumen; P: Plaque; M: Media; A: Adventitia. H&E. Scale bars: 500 µm.

4.36 Three-dimensional scanning electronmicrograph of aorta from CN and HCD groups. Aorta in CN group shows no damage on the smooth intimal surface (I), with regular and clear circular arrangement of VSMCs (M). Aorta from HCD group illustrates many elevated cheesy-like fatty streak masses signify the atherosclerotic plaques (P) that in some area partially detached (yellow arrow). L: Lumen; M: Media; A (dashed yellow arrow): Adventitia. Scale bars: CN: 500 µm; HCD: 200 µm.

4.37 Three-dimensional scanning electronmicrograph of aortas from HCD, ZI, ZII and ZIII groups. Aortas from ZER treatment groups illustrate evident reduction in plaque size (P) (yellow arrows) compare to HCD group. Cross section through atheromas plaque show many empty spaces that stand for empty lipid vacuoles and foam cells. L: Lumen; M: Media; A
Three-dimensional scanning electronmicrograph of aortas in HCD, SG and ZSG groups. Aortas from SG and ZSG groups show pronounced reduction in plaque size (P) (yellow arrow) compared to HCD group, represents by increased number of empty spaces within the plaque (dashed yellow arrow). L: Lumen; M: Media; A: Adventitia. Scale bars: SG: 200 µm; ZSG: 500 µm.

Ultrastructural electronmicrograph of aortic endothelial cell (EC) in CN and HCD groups. CN illustrates normal orientation of the EC on intact internal and external elastic lamellae (black and dashed black arrows). Normal heterochromatic nucleus with nuclear membrane (red arrow) EC lined with intacked cell membrane (dashed red arrow). Yellow arrows represent aortic connective tissue. HCD shows diffuse endothelial cells destruction together with the disruption of basal lamina (yellow arrow). Presence of abundant of electrodense (ED) and electrotranslucent (ET) particles of necrotic debris represent fragmented necrotic remnant distributed randomly in between the internal and external elastic lamella (red arrow). Dashed yellow and red arrows indicate partially damaged external and internal elastic basal lamellae, respectively. L: Lumen. Scale bars: 5000 nm.

Ultrastructural electronmicrograph of aortic endothelial cell (EC) in HCD, ZI, ZII and ZIII groups. ZI illustrates prominent reduction in the intimal degenerative fragments and necrotic cellular debris together with the lessening of intimal lipid deposition (yellow arrows). Partial restoration of external and internal elastic lamellae (dashed yellow and black arrows). ZII demonstrates obvious diminution of foam cells, lipid vacuoles and fragmented cellular debris in tunica media (dashed yellow arrow). Black arrow refers to the fully regenerative internal elastic lamellae with clear demarcation. Dashed black arrow represents fragmented debris of SMCs and elastic fibers. Yellow arrow indicates elastic fiber remnant. ZIII reveals complete disappearing of necrotic cellular debris with clear and fully grown internal elastic lamellae (dashed yellow arrow). Black arrows indicate fragmented elastic fibers in the tunica media. Presence of some lipid droplet in the media (dashed black arrow). Presence of SMCs with heterochromatin nucleus (yellow arrow). EDB: Electrodense bodies of fragmented SMCs scattered randomly in the tunica media. ED: Electrodense necrotic debris of fragmented SMCs in the tunica media. ND: Necrotic debris in the tunica media. SMC: Smooth muscle cell. L: Lumen. Scale bars: HCD and ZI: 5000 nm; ZII: 2000 nm; ZIII: 1000 nm.

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4.42 Bar graph represents effect of ZER and SIM on lipid peroxidation biomarker MDA. The values of MDA show significant dropping in all treatment groups at W14 compare to HCD group, which is more significant (P<0.05) in ZIII and ZSG groups.

4.43 Bar graph represents the effect of ZER and SIM on the antioxidant biomarker SOD. All treatment groups demonstrate significant increment of SOD activity at W14 compare to HCD group, however the increase value is more significant (P<0.05) in ZSG group.

5.1 Bar graph represents the quantification assay of immuno-reactive positive (IPC) and immuno-reactive negative (INC) cells with macrophage biomarker RAM-11. All ZER supplemented groups show significant reduction in the RAM-11 in a dose dependent manner compare to HCD group.

5.2 Photomicrograph of thoracic aorta represents immunohistochemical staining with RAM-11 antibody from CN and HCD groups. CN shows no immunopositive reactive cells (IPC). HCD displays massive and diffuse IPCs within thick rising plaque (P) indicated by deep brownish-stained macrophages (black and yellow arrows). L: Lumen; M: Media; A: Adventitia. Scale bars: 500 µm.

5.3 Photomicrograph of thoracic aortas from HCD, ZPI, ZPII and ZPIII groups represent immunohistochemical staining with RAM-11 antibody. Aortas in ZPI, II and III groups demonstrate significant reduction in plaque size (P) and the intensity of brownish color RAM-11 immunopositive foam cells compare to the thick plaque in HCD (black arrows). Obviously, there is no reaction in the muscularis layer (M). L: Lumen; M: Media; A: Adventitia. Scale bars: 500 µm.

5.4 Bar graph represents the quantification assay of immuno-reactive positive (IPC) and immuno-reactive negative (INC) cells with VSMCs biomarker HHF-35. All ZER supplemented groups show significant reduction in the IPCs HHF-35.
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5.5 Photomicrograph of thoracic aortas from CN and HCD groups represent immunohistochemical staining with HHF-35 alpha actin antibody. The CN group demonstrates no immunopositive reactive cells. The HCD group shows diffuse VSMCs proliferation indicated by significant increase in the intensity of HHF-35 biomarker brownish color in the tunica media (M), with obviously no reaction in the intimal thick plaque (P). L: Lumen; M: Media; Ad: Adventitia. Scale bars: 500 µm.

5.6 Photomicrograph of thoracic aortas from HCD, ZPI, ZPII and ZPIII represent immunohistochemical staining with HHF-35 alpha actin antibody. Aortas from ZPI, II and III groups show significant lessening in the number of immunoreactive positive cells together with the obvious reduction in the density of brown coloration of the tunica media as well as plaque (P) size (black arrows). L: Lumen; M: Media; Ad: Adventitia. Scale bars: 500 µm.

5.7 Bar graph represents the immunoquantitative assay of immunopositive fluorescent cells (IPFC) and immunonegative non-fluorescent cells (INNFC) cells of macrophage-specific protein biomarker RAM-11. Supplemented groups ZPI, II and III show significant P<0.05 reduction in the mean percentage of RAM-11 compare to HCD group.

5.8 Photomicrograph of thoracic aortas from CN and HCD groups symbolize immunoflourescent staining with RAM-11 antibody. Control group shows no immunopositive reaction. HCD group demonstrate pronounced immuno-fluorescent positive reactive cells with increase intensity and percentage of greenish-fluorochrome foam cells in the intimal plaque (P). L: Lumen; M: Media; Ad: Adventitia. Scale bars: CN: 200 µm; HCD: 100 µm.

5.9 Photomicrograph of thoracic aortas from HCD, ZPI, ZPII and ZPIII represent immunoflourescent staining with RAM-11 antibody. Aortas in ZPI, II and III groups demonstrate significant reduction in the macrophage-derived foam cells within the tunica intima compare to HCD group, indicated by low intensity of greenish-fluorochrome immuno-positive RAM-11 cells in the intimal plaque (P) (white arrows). L: Lumen; P: Plaque; M: Media; Ad: Adventitia. Scale bars: 200 µm.

5.10 Bar graph represents the immunoquantitative assay of immunopositive fluorescent cells (IPFC) and immunonegative non-fluorescent cells (INNFC) cells of VSMCs specific protein biomarker HHF-35. All ZER supplemented groups ZPI, II and III show statistically significant P<0.05 reduction in the mean percentage of VSMCs IPFCs in a dose dependant manner compare to HCD group.
5.11 Photomicrograph of thoracic aortas from CN and HCD groups illustrates immunofluorescent staining with HHF-35 antibody. Control group shows no immunopositive reaction in the muscularis layer. Aorta in HCD group reveals distinct immunofluorescent positive reaction with increase intensity and sharpness of greenish-fluorochrome highly proliferative VSMCs within the tunica media (white arrow). L: Lumen; M: Media; Ad: Adventitia. Scale bars: 200 µm.

5.12 Photomicrograph of thoracic aortas from HCD, ZPI, ZPII and ZPIII groups represent immunofluorescent staining with HHF-35 alpha actin antibody. Aortas in ZPI, II and III groups show significant reduction in the percentage of immunoreactive greenish positive VSMCs together with the obvious lessening of greenish-fluorochrome proliferative biomarker compare to HCD group (white arrows). L: Lumen; P: Plaque; M: Media; Ad: Adventitia. Scale bars: 200 µm.

5.13 Bar graph represents the quantification assay of apoptotic cells (APC) and non-apoptotic cells (NAPC) shows significant P<0.05 increase of apoptotic cells in ZER supplemented groups compare to HCD group, the mean percentages of APC are more significant in ZPIII and II, respectively.

5.14 Photomicrograph of thoracic aortas from CN and HCD groups represent in vivo antiproliferative effect of ZER (TUNEL assay). Apparently, there is no or mild apoptotic reaction in CN and HCD aortas, evident by red color nuclei of VSMCs as well as foam cells within the intimal plaque (P) (white arrows). L: Lumen; M: Muscularis layer; Ad: Adventitia. Scale bars: CN: 100µm; HCD: 200µm.

5.15 Photomicrograph of thoracic aortas from HCD, ZPI, ZPII and ZPIII groups represent in vivo antiproliferative effect of ZER (TUNEL assay). Aortas in ZPI, II and III groups show significant augmentation in the number of apoptotic cells in both intimal plaque (P) and muscularis (M) evidently with increase in greenish-yellow to orange-color glistening apoptotic nuclei (white arrows) disseminated throughout the aortic sections. L: Lumen; M: Muscularis layer; Ad: Adventitia. Scale bars: 100 µm.

5.16 Western blot image analysis from aortic tissue by chemiluminescent gel doc for NF-κB, Cox-2, and iNOS. All ZER supplemented groups show significant reduction in the protein band thickness, which indicates downregulation of protein expression, particularly in ZPIII which is highly significant.

5.17 Bar graph represents protein transcription analysis in aortic tissue by Western blotting assay using Image J software. Data reveal significant (P<0.05) suppression of NF-κB, Cox-2, and iNOS proteins in ZER preventive groups compare to that of HCD group, in which the effect is more significant in ZPIII.
Western blot image analysis from aortic tissue by chemiluminescent gel doc for Bax and Bcl-2. Zerumbone supplementation significantly upregulate proapoptotic Bax expression and down regulate antiapoptotic Bcl-2 protein expression in dose dependent manner, indicated by thick bands in Bax and very thin bands in Bcl-2.

Bar graph represent protein transcription analysis in aortic tissue by Western blotting assay using Image J software. Data show significant (P<0.05) downregulation of Bcl-2 and up-regulation of Bax proteins ZP groups, compare to HCD group. The effect is more significant in ZPIII and II, respectively.

Bar graph represent serum proinflammatory cytokines (TNF-α, IFN-γ, IL-1, and IL-6) analysis by ELISA assay. Data reveal significant (P<0.05) reduction in the serum inflammatory cytokines in all ZER supplemented groups in a dose dependent manner compare to HCD group. However, more statistically significant reduction can be seen in ZPIII group.

Bar graph represents the quantification assay of immuno-reactive positive (IPC) and immuno-reactive negative (INC) cells with macrophage-specific protein biomarker RAM-11. All treatment groups demonstrate statistically significant reduction in RAM-11 expression compare to HCD group.

Photomicrograph of thoracic aortas from CN and HCD groups represent immunohistochemical staining with RAM-11 antibody. Control group shows no immuno-reactive positive cells (IPC). Aorta in HCD group present diffuse IPCs within thick rising plaque (P) indicated by deep brownish-stained macrophages-derived foam cells. L: Lumen; M: Media; Ad: Adventitia. Scale bars: 200 µm.

Photomicrograph of thoracic aortas from HCD, ZI, ZII and ZIII groups represent immunohistochemical staining with RAM-11 antibody. Aortas in ZI, II and III groups reveal significant reduction in plaque size (P) (black arrows) and the intensity of brownish color of immunopositive foam cells within the tunica intima compare to that of HCD group. Obviously, there is no reaction in the muscularis layer (M). L: Lumen; M: Media; Ad: Adventitia. Scale bars: 200 µm.

Photomicrograph of thoracic aorta from SG and ZSG groups represent immunohistochemical staining with RAM-11 antibody. Aortas in SG and ZSG groups reveal reduction in RAM-11 immunopositive reactive cells compare to HCD group, however ZSG group shows more significant reduction in macrophage expression within the intimal plaque (black arrows), indicated by profound lessening in the reactive brownish staining intensity. L: Lumen; M: Media; Ad: Adventitia; P: Plaque. Scale bar: 200 µm.

Bar graph represents the quantification assay of immuno-
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5.26 Photomicrograph of thoracic aortas from CN and HCD groups represent immunohistochemical staining with HHF-35 alpha actin antibody. Aorta in CN demonstrates no immuno-reactive cells in the tunica media. Aorta in HCD displays diffuse VSMCs proliferation evident by significant increase in the intensity of HHF-35 biomarker brownish color in the tunica muscularis, with obviously no reaction in the intimal thick plaque (P). L: Lumen; M: Media; Ad: Adventitia. Scale bars: 200 µm.

5.27 Photomicrograph of thoracic aortas from HCD, ZI, ZII and ZIII represent immunohistochemical staining with HHF-35 alpha actin antibody. All aortas in treatment groups show significant lessening in the number of immunoreactive positive cells (IPCs) together with the obvious reduction in brownish stain intensity of the tunica media as well as plaque size (black arrow) compare to HCD group. L: Lumen; M: Media; Ad: Adventitia. Scale bars: 200 µm.

5.28 Photomicrograph of thoracic aorta from SG and ZSG groups represent immunohistochemical staining with HHF-35 antibody. Aorta in ZSG group shows significant lessening in the intensity of immunoreactive brownish positive VSMCs biomarker HHF-35 in the tunica media. In SG group less significant reduction in HHF-35 reactive cells is observed than in ZSG group. However, both groups display anti HHF-35 expression compare to HCD group. L: Lumen; M: Media; Ad: Adventitia. Scale bars: 200 µm.

5.29 Bar graph represents the immunoquantitative assay of immunoposative fluorescent cells (IPFC) and immunonegative non-fluorescent cells (INNFC) cells of macrophage RAM-11 biomarker. All treatment groups show statistically significant P<0.05 reduction in the percentage of RAM-11 IPFCs in a dose dependant manner compare to HCD group.

5.30 Photomicrograph of thoracic aortas from CN and HCD groups represent immunoflourescent staining with RAM-11 antibody. Aorta in CN group shows no immunoposative reaction with RAM-11. Aorta in HCD group reveals significant immunopositive reactive RAM-11 cells with increase intensity of greenish-fluroochrome area in the intimal plaque (P) (white arrow). L: Lumen; M: Muscularis layer (Media); Ad: Adventitia. Scale bars: CN: 200 µm; HCD: 100 µm.

5.31 Photomicrograph of thoracic aortas from HCD, ZI, ZII and ZIII groups represent immunoflourescent staining with RAM-11 antibody. Aortas in ZI, II and III demonstrate significant lessening in the formation of foam cells-macrophage origin in
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5.32 Photomicrograph of thoracic aorta from SG and ZSG groups represent immuno-fluorescent staining with RAM-11 antibody. Aorta in SG group demonstrates less significant reduction in RAM-11 immunopositive reactive area compare to ZSG group within the intimal plaque (P) (white arrows). However, ZSG group shows more significant lessening in the intensity and percentage of immuno-fluorescent positive area compare to HCD group. L: Lumen; M: Media. Scale bars: HCD and SG: 100 µm; ZSG: 200 µm.

5.33 Bar graph represent the immunoquantitative assay of immunopositive fluorescent cells (IPFC) and immunonegative non-fluorescent cells (INNFC) cells of VSMCs HHF-35 biomarker. All treatment groups demonstrate statistically significant reduction in the percentage of VSMCs IPFCs in a dose dependant manner compare to HCD group.

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5.35 Photomicrograph of thoracic aortas from HCD, ZI, ZII and ZIII groups represent immuno-fluorescent staining with HHF-35 alpha actin antibody. All treatment groups demonstrate significant reduction in the immunoreactive greenish positive VSMCs in a dose dependant manner compare to HCD group, indicated by obvious attenuation in the area of greenish-fluorochrome proliferative biomarker within the muscularis layer (white arrows). L: Lumen; P: Plaque; M: Media; Ad: Adventitia. Scale bars: 200 µm.

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5.40 Photomicrograph of thoracic aorta from SG and ZSG groups represents in vivo antiproliferative effect of ZER (TUNEL) assay. Aorta in ZSG group shows much significant increase in the number of greenish-yellow to orange-color glistening apoptotic nuclei in both intimal plaque and muscularis layer (white and dashed arrows) compare to HCD and SG groups which show much mild apoptotic reaction within the media. L: Lumen; P: Plaque; M: Media. Ad: Adventitia. Scale bars: 100 µm.

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5.42 Bar graph represent protein transcription analysis in aortic tissue by Western blotting assay using Image J software. Data show significant (P<0.05) suppression of NF-κB, Cox-2, and iNOS proteins in treatment groups compare to HCD group, in which the values are more significant in ZIII and ZSG groups.

5.43 Western blot image analysis from aortic tissue by chemiluminescent gel doc for Bax and Bcl-2 proteins. All ZER treatment groups reveal significant up-regulation of Bax and down regulation of Bcl-2 proteins expression compare to HCD group, yet the results are more significant in ZIII and ZSG groups.

5.44 Bar graph represent protein transcription analysis in aortic tissue by Western blotting assay using Image J software. Data reveal significant (P<0.05) suppression of Bcl-2 and over-
expression of Bax protein in all ZER treatment groups compare to HCD group. However, the results are more significant in ZIII and ZSG groups.

Bar graph represent serum proinflammatory cytokines (TNF-α, IFN-γ, IL-1, and IL-6) analysis by ELISA assay. Data reveal significant (P<0.05) reduction in the serum proinflammatory cytokines in all treatment groups in a dose dependant manner compare to that of HCD group, which is more significant in ZSG group.
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>®</td>
<td>Trade Mark</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<tr>
<td>µl</td>
<td>Microlitre</td>
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<td>µm</td>
<td>Micro Meter</td>
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<tr>
<td>2X</td>
<td>Two Fold</td>
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<td>Å</td>
<td>Angstrom</td>
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<tr>
<td>AA</td>
<td>Arachidonic acid</td>
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<tr>
<td>ACS</td>
<td>Acute Coronary Syndrome</td>
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<tr>
<td>ACUC</td>
<td>Animal Care and Use Committee</td>
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<tr>
<td>ADP</td>
<td>Adenosine Di Phosphate</td>
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<td>AGE</td>
<td>Advanced Glycation End Products</td>
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<tr>
<td>Alb</td>
<td>Albumin</td>
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<tr>
<td>ALL</td>
<td>Acute Lymphocytic Leukemia</td>
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<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
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<tr>
<td>ALT</td>
<td>Alanine Aminotransferase</td>
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<td>Ang-II</td>
<td>Angiotensin-II</td>
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<tr>
<td>ANOVA</td>
<td>One-Way Analysis of Variance</td>
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<tr>
<td>apoA1</td>
<td>Apolipoprotein A1</td>
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<tr>
<td>AST</td>
<td>Aspartate Aminotransferase</td>
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<tr>
<td>Bax</td>
<td>Becl2 Associated X Protein</td>
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<tr>
<td>BDMA</td>
<td>Benzyl Dimethyl Amine</td>
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<tr>
<td>BHT</td>
<td>Butylated Hydroxytoulene</td>
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<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
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<tr>
<td>CHD</td>
<td>Coronary Heart Diseases</td>
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<tr>
<td>Cm</td>
<td>Centimeter</td>
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<tr>
<td>cm²</td>
<td>Square Centimetre</td>
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<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
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<tr>
<td>CPD</td>
<td>Critical Point Drier</td>
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<tr>
<td>Creat</td>
<td>Creatinine</td>
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<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
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<tr>
<td>Cu Kα</td>
<td>Copper Anode</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
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<tr>
<td>CXCR3</td>
<td>Chemokine Receptor Type 3</td>
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<tr>
<td>Cyt-c</td>
<td>Cytochrome C</td>
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<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
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<tr>
<td>DCs</td>
<td>Dendritic Cells</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide</td>
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<tr>
<td>DPX</td>
<td>Mounting Media And Section Adhesive</td>
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<tr>
<td>EDTA</td>
<td>Ethyl Diamine Tetra Acetic Acid</td>
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<tr>
<td>EE</td>
<td>Ethanolic Extract</td>
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<tr>
<td>EEZZ</td>
<td>Ethanolic Extract of Zingiber Zerumbet Rhizome</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbant Assay</td>
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<tr>
<td>EM</td>
<td>Electron Microscopy</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factors</td>
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<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<tr>
<td>g</td>
<td>Gram</td>
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</table>
GGT  \(\Gamma\)-Glutamyl Transferase
GSH  Glutathione
h  Hour (S)
H&E  Haematoxylin And Eosin
HCD  High-Cholesterol Diet
HDL  High-Density Lipoprotein
HMG-CoA  3-Hydroxy-3-Methyl Glutaryl Coenzyme A
HPLC  High Performance Liquid Chromatography
HRP  Horse Radish Peroxidase
ICAM-1  Intercellular Adhesion Molecule 1
IDL  Intermediate Density Lipoprotein
IFA  Immunofluorescent Assay
IFN-\(\gamma\)  Interferon-\(\gamma\)
IHC  Immunohistochemistry
IL-1  Interleukin-1
IL-6  Interleukin-6
iNOS  Inducible Nitric Oxide Synthase
IkB\(\alpha\)  Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Alpha
\(\kappa\)  Kappa
kDa  Kilo Dalton
Kg  Kilogram
KH\(_2\)PO\(_4\)  Potassium Dihydrogen Phosphate
L  Litre
LOX-1  Lectin-Type Oxidized LDL Receptor 1
MCP-1  Monocyte Chemotactic Protein 1
MDA  Malondialdehyde
MeOH  Methanol
mg  Milligram
MI  Myocardial Infarction
min  Minute
mL  Millilitre
MM  Mucus Membrane
Mm  Micromolar
mm  Millimetre
MMP  Matrix Metalloproteinases
n  Number
NaCN  Sodium Cyanide
NADPH  Nicotinamide Adenine Dinucleotide Phosphate
NaOH  Sodium Hydroxide
NF-\(\kappa B\)  Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NH\(_4\)Cl  Ammonium Chloride
nmol  Nanomole
NMR  Nuclear Magnetic Resonance
NO  Nitric Oxide
OD  Optical Density
Ox-LDL  Oxidized Low Density Lipoprotein
\(P < 0.05\)  Probability Values of Less Than Alpha 0.05
PBS  Phosphate Buffer Saline
PDGF  Platelet-Derived Growth Factor
PGE2  Prostaglandin E2
pH   Measurement For Hydrogen Ion Concentration
PI   Propidium Iodide
PPAR-α Peroxisome Proliferator-Activated Receptor-Alpha
RNAase Ribonuclease Enzyme
ROS  Reactive Oxygen Species
rpm Revolution Per Minute
SDS- Sodium Dodecyl Sulphate-Polyacrylamide Gel
PAGE Electrophoresis.
Sec Second (S)
SEM Scanning Electron Microscope
SMC Smooth Muscle Cells
SOD Superoxide Dismutase
SPSS Statistical Package For The Social Sciences
T-cell T Lymphocyte
TEM Transmission Electron Microscopy
TNF-α Tissue Necrotizing Factor-Alpha
TUNEL Tdt-Mediated Dtp Nick-End Labelling
UPM University Putra Malaysia
v/v Volume To Volume
VCAM-1 Vascular Cell Adhesion Molecule-1
VEGF Vascular Endothelial Growth Factor
VLDL Very Low-Density Lipoprotein
VSMC Vascular Smooth Muscle Cells
w/v Weight To Volume
WB Western Blotting
WHHR Watanabe Heritable Hyperlipidemic Rabbit
WHO World Health Organization
ZER Zerumbone
ZZ Zingiber Zerumbet (L.) Smith
β-actin Beta Actin
γ Gamma
CHAPTER ONE

GENERAL INTRODUCTION

Atherosclerosis is a complex, chronic, proliferative and accumulative inflammatory disorder with the involvement of immune system (Ross, 1999; Galkina and Ley, 2009). It is a disease of large and medium sized arteries characterized by focal intimal thickening of the arterial wall associated with lipid deposition in the form of elevated lipid-filled plaques called atheromas (Ross et al., 1984). Necrosis and fibrosis that follow the fibro-fatty plaque progression probably will result in partial or complete occlusion leading to ischemia and infarction (Ross, 1993b). It is of greatest importance of cardiovascular disease (CVD) in humans and is considered a public health issue accounting for an estimated 50% of overall deaths in western countries (O’Connor et al., 2001; Stocker and Keaney, 2004).

It is generally believed that atherosclerosis is a chronic inflammatory response that is advanced by lifestyle-related disorders, such as elevated serum cholesterol particularly low density lipoprotein (LDL-cholesterol), hypertension, diabetes mellitus and cigarette smoking (Altman, 2003; Sata and Fukuda, 2011). Recently hyperhomocysteinemia (Faraci and Lentz, 2004) and infectious microorganisms such as Cytomegalovirus, herpesviruses, Helicobacter pylori and Chlamydia pneumoniae (Chiu, 1999; Ameriso et al., 2001) are believed to be contributors to the initiation and development of atherosclerosis.

Numerous pathophysiological investigations in humans and animals led to the formulation of the response-to-injury hypothesis of atherosclerosis (Ross and Glomset, 1973), which principally proposed that endothelial denudation was the initial step in the process of atherogenesis (Ross and Glomset, 1976). This postulation supports the early theory suggesting that cellular responses in atherosclerosis are secondary in response to mechanical and/or toxic injuries leading to endothelial dysfunction (Von Rokitansky and Swaine, 1855). This is in contrast, to the postulated an initial and critical role of cellular pathology in the formation and development of atherosclerosis (Virchow, 1860), once more supported by Ross (Ross, 1999) and antithetical to the humoral pathology theory of the Rokitansky’s school (Mayerl et al., 2006).

Currently, large number of recent work emphasize that the chronic inflammatory reaction together with involvement of innate and adaptive immune response in association with the traditional risk factors play a pivotal role in the initiation and progression of atherosclerosis (Hansson and Libby, 2006; Kaperonis et al., 2006; Libby, 2012). Atherogenesis comprises three fundamental stages, including intimal thickening, plaque development, and plaque destabilization-rupture (Sakata, 2012). The initial and earliest lesion of atherosclerosis the so-called fatty streaks (Ross et al., 1984) is common in infant and young children (Napoli et al., 1997). It is merely and simply an inflammatory lesion consisting of T-lymphocytes (T-cells), dendritic cells (DCs), and monocyte-deriving macrophages, with involvement of innate and adaptive immune systems (Hansson and Libby, 2006; Galkina and Ley, 2009).
As fatty streaks developed, in result of endothelial injury that accumulates lipid, extracellular lipid particularly low density lipid (LDL) modified by oxidative pathway and engulfed by macrophages to form immobile foam cells (Stary et al., 1994). Cytokines, growth factors, adhesion molecules and chemotactic proteins are released by chronic inflammatory cells and denuded endothelial cells result in monocytes recruitment, extracellular matrix production and smooth muscle cells (SMCs) proliferation-migration and transformation to foam cells in the intima (Lusis, 2000).

Foam cells originated from transformed SMCs that are subjected to death by apoptosis (Okura et al., 2000). Progression of atherosclerosis from early to advance lesions will initiate with the generation of lipid-rich core in the deep layer of thickened intima derived from dead foam cells containing necrotic tissues and free cholesterol crystals which is called atheroma, that wrapped by thick fibrous cap (Ross, 1993a). Continuous atheroma build up result in arterial luminal narrowing particularly in the coronary artery, and it is considered a primary cause of stable angina pectoris (Sakata, 2012).

Persistent recruitment of inflammatory cells into the lesion particularly macrophages render the fibrous cap thin and make the plaque weak in architecture (Lendon et al., 1991), therefore, more vulnerable to rupture in response to the physical forces of blood flowing causes ulceration, hemorrhage, and thrombus formation, which result in sudden death from myocardial infarction and stroke (Ross, 1999). Coronary artery disease (CAD) arising from atherosclerosis is a leading cause of mortality worldwide. Currently available therapeutics against atherosclerosis is basically limited to alleviating the traditional risk factors such as hyperlipidemia and hypertension or controlling the thrombotic complications (Weber and Noels, 2011).

Treatment with statin drugs in patients with CAD shows a significant reduction in risk factors that is correlates to LDL-cholesterol, thereby limiting plaque development and reduces possibly of plaque rupture throughout its pleiotropic anti-inflammatory and antihypercholesterololaemic effects, endothelial dysfunction improvement, and reducing thrombogenicity (Ray and Cannon, 2005). Furthermore, monitoring high blood pressure with anti hypertensive beta-blockers contributes to lower mortality from myocardial infarction and stabilized atheroprogression (Sipahi et al., 2007). More recently, medication with artificial peptide-based high density lipoprotein (HDL)-like apolipoprotein as an additive in statin-treated patients exert a profound anti-inflammatory and lipid lowering effects (Navab et al., 2010).

On the other hand, concerning the dangerous side effects of these chemical remedies such as high doses of statins in patients with high serum cholesterol have been found to cause striated muscle damage (Antons et al., 2006), and increase the risk of rhabdomyolysis associated with neuropathy (Fadini et al., 2010). In addition to the complicated nature and pathogenesis of atherosclerosis, involving oxidative stress damage, endothelial dysfunction (Victor et al., 2009), elevated level of LDL-cholesterol and chronic inflammatory reaction (Libby et al., 2002; Kaperonis et al., 2006).
As a result, seeking a multifaceted natural product-based complementary and alternative herbal medicine, which is readily available, effective, and addressed all major risk factors with no toxic effects, was the best choice in providing an ideal remedy in the prevention and treatment of atherosclerosis (Zeng et al., 2012).

The usage, interest, and self-administration of herbal medicine are widespread and most popular alternative therapy among patients under CVDs pharmacotherapy (Izzo et al., 2005). Using natural compounds as a supplementary healthy diet is not a substitute for regular medical care, however, it is considered as a complementary and alternative medicine (Brown et al., 2007). Medical herbs and plant foods such as fruits, vegetables, and spices contain many biologically active phytochemical compounds that have various health promoting effects (Lampe, 1999).

*Zingiber zerumbet* (L.) Smith belonging to Zingiberaceae, is an edible ginger, originating from South-East Asia has been cultivated thousands of years as a spice-food additive and for medical purposes (Vimala et al., 1999). Whereas, approximately 161 species from 18 genera of this family are found in Peninsular Malaysia (Ruslay et al., 2007). The extracts of *Zingiber zerumbet* rhizomes, have been used as a traditional medicine to treat various types of inflammatory mediated human ailments as a potent inflammatory suppressor contribute to down regulate the proinflammatory mediators such as prostaglandin E2 (PGE2) (Chien et al., 2008).

Recently, the rhizome’s extract have been extensively studied in multiple investigations for its effectiveness in a broad range of biological activities include antinociceptive (Sulaiman et al., 2009), anti-inflammatory (Zakaria et al., 2010), antioxidant (Yob et al., 2011), antimicrobial (Habsah et al., 2000), antifungal (Jantan et al., 2003), antitumor (Kirana et al., 2003) and antiplatelet aggregation (Jantan et al., 2008). More recently, ethanolic extract of *Zingiber zerumbet* showed antihypercholesterolaemic property in rats fed a high-fat diet (Chang et al., 2012).

Of all bioactive compound(s) isolated and identified from various extracts of *Zingiber zerumbet* rhizomes, zerumbone has been studied extensively due to its broad-spectrum biomedical properties. Zerumbone (ZER) is a crystalline, monocyclic, sesquiterpene, phytochemical substance that was first isolated as a major compound in 1960 from the essential volatile oil of rhizomes of *Zingiber zerumbet* (L.) smith (Kitayama et al., 2003). It predominantly can be isolated from both leaves and rhizomes of the plant at approximately 36.98% and 46.83%, respectively (Bhuiyan et al., 2008).

Numerous biological and therapeutical activities of ZER includes anticancer and antioxidant (Murakami and Ohigashi, 2006), anti-inflammatory (Somchit et al., 2012), antinociceptive (Zakaria et al., 2010), antimicrobial (Kader et al., 2011), hepatoprotective (Fakurazi et al., 2009), antiproliferative and apoptosis inducing agent (Sakinah et al., 2007b) and immunomodulatory activities (Keong et al., 2010) in a dose dependant manner. In addition, ZER has been demonstrated to attenuate inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression via modulation of nuclear factor kappa-B cells (NF-κB) activation (Takada et al., 2005; Murakami and Ohigashi, 2007), beside its potent anti-inflammatory (Sulaiman et al., 2010) and antioxidant (Murakami et al., 2003) efficacy.
Additionally, antiproliferative and apoptotic inducing effects of ZER been approved against variety of organ carcinogenesis via down and up regulation of Bcl-2 and Bax, respectively (Murakami et al., 2002; Takada et al., 2005; Sakinah et al., 2007a). Moreover, ZER evidently approved to suppress some cytokines that play a major role in the process of atherogenesis such as interleukin-6 (IL-6) (Abdelwahab et al., 2012) and tissue necrotizing factor-alpha (TNF-α) (Chen et al., 2011).

Depend upon the mentioned phytotherapeutic effects of ZER against proliferative and inflammatory diseases via suppression of proinflammatory mediators and cytokines (Sulaiman et al., 2010). In addition to its active antioxidant property in suppressing free radicals generation and its potent antiproliferative activity via upregulation of proapoptotic genes (Murakami et al., 2002). Furthermore, to date, no study has addressed the effect of ZER on serum lipid profile in relation to atherosclerosis progression in rabbits. Therefore, the general objective of this study is to investigate the prophylactic and therapeutic efficacies of dietary ZER supplementation on the development of atherosclerosis in rabbits fed with high cholesterol diet.

**Problem Statement**

Difficulty in finding a relatively efficacious, non-toxic, readily available, cheap, and naturally existing antiatherogenic and antihyperlipidaemic agent warrants the search for such an agent.

**Hypothesis**

**Null Hypothesis**

Zerumbone supplementation shows no lipid lowering effect and not an antiatherogenic agent, thus will not prevent and reduce the early development of atherosclerotic lesions in hypercholesterolemic rabbits.

**Alternative or Research Hypothesis**

Zerumbone will prevent and reduce the development of atherosclerotic plaques induced by high cholesterol diet via suppression and down regulation of proinflammatory mediators and cytokines thus inflammatory reaction. As well as, inducing apoptosis and reducing smooth muscle cell proliferation-migration in turn plaque propagation. Finally yet importantly, suppressing free radicals production hence, minimizing oxidative stress damage, and lowering lipid profile subsequently alleviate plaque development.
**Aim and objectives**

The main aim of the study is to evaluate the prophylactic and therapeutic efficiencies of ZER supplementation on the formation, development, and establishment of early atherosclerosis in rabbits fed with high-cholesterol diet with the following objectives:

1) To evaluate the antihypercholesterolaemic effect of dietary ZER supplementation on the initiation and propagation of atherosclerosis in cholesterol-fed rabbits.

2) To estimate the antioxidant efficacy of dietary ZER supplementation on the formation and development of atherosclerosis in hypercholesterolemic rabbits.

3) To assess the anti-inflammatory effect of ZER supplementation on the initiation and development of atherosclerosis in rabbits fed high-cholesterol diet.

4) To estimate the antiproliferative and apoptosis inducing effect of ZER supplementation on the formation and progression of atherosclerosis in rabbits on high-cholesterol diet.
REFERENCES


