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ORIGINAL ARTICLE

HISTO-INHIBITORY EFFECTS OF WARBUGIA UGANDENSIS ON HIGH FAT DIET INDUCED ATHEROSCLEROSIS IN NEW ZEALAND RABBITS

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ABSTRACT

Atherosclerosis is a chronic inflammatory condition characterized by accumulation of plaque within a blood vessel. *W. ugandensis* has antioxidant and anti-inflammatory benefits therefore it might play an important role in histo-inhibition of atherosclerosis. This was a posttest only true experimental design in which 30 male New Zealand rabbits were used. Systematic random sampling method was used in recruiting and assigning the animals into control and experimental groups. *W. ugandensis* extract was obtained, phytochemical analysis and acute oral toxicity was done to determine safe dose. The animals were fed on high fat diet to induce atherosclerosis. The mean area fraction of Atorvastatin and *W. ugandensis* histo-inhibitory group significantly reduced as compared to vehicle control group. On histological analysis, histo-inhibitory group had a fatty streak within the tunica intima characterized by foam cells that accumulated lipids in their cytoplasm while the atorvastatin histo-inhibitory group had less pronounced fatty streak. Therefore, it can be concluded that *W. ugandensis* has positive histo-inhibitory effects characterized by failure of formation of atherosclerotic lesion.

Keywords: Antioxidant, anti-inflammatory, Atorvastatin, Histo-inhibition and phytochemicals.
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INTRODUCTION

Typically, a histo-inhibitory agent works to stop atherosclerosis from developing. This calls for the use of both protecting and harmful agents in combination. After which, the effect is assessed histologically. Atherosclerosis is initiated by sub endothelial injury that causes inflammation and infiltration of LDL which becomes oxidized and forms a fatty streak as initial stage. According to (Castro *et al., 2009)* studies reveal that oxygen reactive species contribute to atherogenesis and cardiovascular disease progression, while exposure to low-density lipoproteins and nucleic acids can lead to harmful oxidative changes. (Kollár & Hotolová, 2003) oxidative theory suggests that excessive LDL oxidation promotes atherosclerosis, as native LDL isn't pathogenic.

Antioxidants reduce lesions formation, suggesting lipid oxidation plays a role in atherogenesis. Research conducted by (Khera *et al.*, 2020) indicates that *W. ugandensis* possesses exceptional anti-inflammatory and antioxidant properties, especially after the identification of a novel aryl naphthalene lignan amide. It is also cheap, versatile and readily available in the local markets which might be very beneficial to the population using it.

In a study done by (Mandlik & Namdeo, 2021) on atherosclerosis of middle cerebral artery, it was discovered that the lesion area significantly reduced secondary to use of herbal plant with high antioxidative and anti-inflammatory benefits. The presence of antioxidant and anti-inflammatory phytochemicals in *Warbugia ugandensis* has been shown to have a histo-inhibitory effect, which means that although the lesion may not proceed to the point of plaque formation,

fatty streaks and other early stages of atherosclerosis may be visible.

MATERIALS AND METHODS

Experimental design and animals

This was a posttest only true experimental design which involved use of 30 male New Zealand rabbits calculated using resource method formula. In this case posttest study design was adopted because the study involved inducing atherosclerosis and giving *W. ugandensis* then results were compared. Systematic random sampling method was used in recruiting and assigning the animals into control and experimental groups, this method was adopted as it has minimal bias given that all animals have an equal chance of being included in the study.

Drug preparation and administration

W. ugandensis stem barks were collected randomly with assistance of a plant taxonomist from Mount Kenya forest. They were then washed, air dried and grounded into powder and stored in plastic bags until extraction. 1000grams of plant powder was soaked in room temperature for one day then filtered with Whitman no.1 filter paper after which solvent evaporation was achieved. Phytochemical components were determined, qualitative analysis was done and acute oral toxicity conducted to determine the safe dose of extract.

To induce atherosclerosis, animals were fed on 50g of high fat diet (1.5% cholestrol) for seven weeks (Qiao *et al*., 2017) in positive control. Atorvastatin was given concurrently with high fat diet while experimental groups received W. ugandensis extract concurrently with high fat diet to achieve histo-inhibitory effects. During this process high hygiene standards and animal handling occupational procedures were adhered to strictly throughout the study.

Sacrificing of animals

Concentrated carbon iv oxide was used to sacrifice the animals at the end of week fifteen. A mid line incision was made from jugular notch to pubis symphysis to expose abdominal-pelvic viscera. The heart was identified, perfusion with 10% formalin done, then heart and aorta were dissected. The aorta was then preserved in 4% formalin. Staining of atherosclerotic lesion was done using oil red O working solution. Aortic tissue fixed by Bousin`s solution for 24 hours. Later the slides were stained in hematoxylin eosin solution for light microscopy.

Photomicrograph were obtained using Leica M125 stereomicroscope mounted with DFC450 camera at magnification of x8. The area fraction of aortic intima was done using image J, image analysis software. Data on aortic intima fraction was analyzed using SPSS version 26.0, one way ANOVA was adopted to determine mean difference and later subjected to post hoc Bonferroni test. A significance level of $p<0.05$ was considered significant. The ethical approval was adopted from JKUAT(JKU/ESRC/02316/0891) and NACOSTI (NACOSTI/P/23/28152).

RESULTS

Determination of the histo-inhibitory effects of W. ugandensis on an atherosclerotic lesion of white New Zealand rabbits.

The mean area fraction of vehicle control group significantly ($p=0.0001$) increased when compared to negative control group at $0.51622mm^2$ and $0.15144mm^2$ respectively (Table 1).

Table 1: Comparison of mean fraction area between control groups.

GROUPS					
	Negative Control (water + food)	Vehicle Control (DMSO)	df	F	p value
AREA FRACTION (mm^2)	0.15144 ±.02	0.51622± .36	5	86.493	0.0001*

All values are expressed and presented as the mean± the standard error of the mean; n=3. Data analyzed by one-way analysis of variance followed by post-hoc Bonferroni. Asterisks represents significant ($p \leq 0.0001$).*

Vehicle control versus *W. ugandensis* inhibitory groups

The mean area fraction for *W. ugandensis* histo inhibitory group significantly (p=0.0001) reduced when compared to vehicle control group at 0.45584mm^2 and 0.51622mm^2 respectively.

Table 2: Comparison mean fraction area between vehicle control and W. ugandensis inhibitory groups.

GROUPS					
	Vehicle Control (High fat diet-50g/day + DMSO)	*W. ugandensis* histoinhibitory (high fat diet-50g/day + *W ugandensis* 500mg/kg/day)	df	F	p value
Area fraction(mm^2)	0.51622 ±.36	0.45584 ±.06	5	86.493	0.0001*

All values are expressed and presented as the mean± the standard error of the mean; n=3. Data analyzed by one-way analysis of variance followed by post-hoc Bonferroni. Asterisks represents significant ($p \leq 0.0001$).*

Vehicle control vs Atorvastatin inhibitory groups

The mean fraction area of atorvastatin group significantly (p=0.0001) reduced at 0.16821mm^2 as compared vehicle group of 0.51622mm^2.

Table 3: Comparison of mean fraction area between vehicle control and atorvastatin inhibitory groups

GROUPS					
	Vehicle Control (High fat diet-50g/day + DMSO)	Atorvastatin histoinhibitory (high fat diet-50g/day + atorvastatin)	df	F	p value
Area fraction(mm^2)	0.51622± .36	0.16821 ± .35	5	86.493	0.0001*

All values are expressed and presented as the mean± the standard error of the mean; n=3. Data analyzed by one-way analysis of variance followed by post-hoc Bonferroni. Asterisks represents significant ($p \leq 0.0001$).*

Negative control vs *W. ugandensis* inhibitory groups

There was no significant (p=1.000) difference of mean area fraction of Warbugia inhibitory group when compared with negative control group.

Table 4: Comparison of mean fraction area between W. ugandensis inhibitory and negative control groups.

GROUPS					
	Negative Control (Feeds + water)	*W ugandensis* Histoinhibitory (high fat diet-50g/day + *W ugandensis* 500mg/kg/day)	df	F	p value
Area fraction(mm^2)	0.15144 ±.02	0.45584 ±.30	5	86.493	1.000

All values are expressed and presented as the mean± the standard error of the mean; n=3. Data analyzed by one-way analysis of variance followed by post-hoc Bonferroni.

Negative control vs Atorvastatin inhibitory groups

There was no significant difference of mean area fraction of negative control group when compared with Atorvastatin inhibitory group (p=1.000).

Table 5: Comparison of mean fraction area between negative control and atorvastatin inhibitory groups.

GROUPS					
	Negative Control (Feeds + water)	Atorvastatin histoinhibitory (high fat diet-50g/day + Atorvastatin)	df	F	p value
Area fraction(mm^2)	0.1514 ±0.2	0.1682± 0.2	5	86.493	1.000

KEY: All values are expressed and presented as the mean± the standard error of the mean; n=3. Data analyzed by one-way analysis of variance followed by post-hoc Bonferroni.

Warbugia Ugandensis **inhibitory VS Atorvastatin inhibitory groups**

There was a significant increase in the area fraction in *W ugandensis* inhibitory group (high fat diet-50g/day + Wu-500mg/kg/day) compared to atorvastatin inhibitory group (high fat diet-50g/day + 1.5mg/kgbwt atorvastatin) which recorded a mean of 0.45584mm and 0.16821mm respectively. A statistical significant difference ($p<0.005$) was observed between *Warbugia ugandensis* inhibitory group compared to atorvastatin inhibitory group.

Table 6: Comparison mean fraction area between experimental groups

GROUPS					
	Atorvastatin histoinhibitory (high fat diet-50g/day + Atorvastatin)	*W ugandensis* histoinhibitory (high fat diet-50g/day + *W ugandensis*-500mg/kg/day)	df	F	p value
AREA FRACTION (mm^2)	0.16821 ± .35	0.45584 ± .29	5	86.493	0.0001*

KEY: All values are expressed and presented as the mean± the standard error of the mean; n=3. Data analyzed by one-way analysis of variance followed by post-hoc Bonferroni. Asterisks represents significant ($p \leq 0.0001$).*

Histological effects of different histo-inhibitory groups

Comparison between negative control and vehicle control groups

It was observed that the negative control group had a well distributed simple squamous endothelial cells lining the endothelium with the sub endothelium showing sparse nucleus and matrix whereas

vehicle control group had large tunica intima, evident lipid core lesion with poorly distributed cells, a necrotic area and a fibrous cap (Figure1).

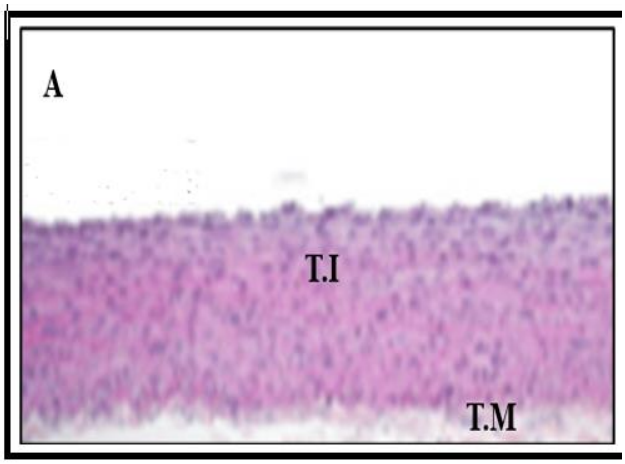

Figure 1: *Showing A= negative control group (feeds + water ad libitum) and B= vehicle control group (high fat diet + 5% DMSO). KEY: T.I =tunica intima, T.M= tunica media*

Comparison of aortic tunica intima between controls and experimental histo-inhibitory groups.

It was observed that *W. ugandensis* histo-inhibitory group had a fatty streak within the tunica intima characterized by foam cells that accumulated lipids in their cytoplasm. On the other hand, Atorvastatin histo-inhibitory group had less pronounced fatty streak (Figure 2).

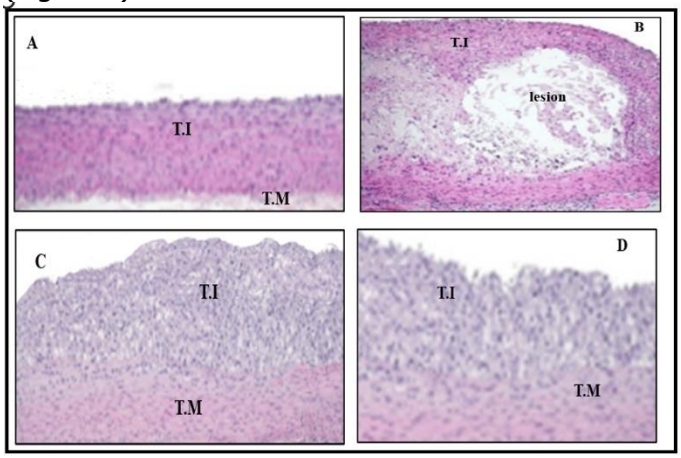

Figure 2: *Showing A= negative control group, B= vehicle control group, C= W. ugandensis histo-inhibitory group and D= Atorvastatin histo-inhibitory group.KEY: TM=tunica media and TI= tunica intima*

DISCUSSION

Histo-inhibition is simply the prevention of changes in histological make up of tissues as a result of use of drug or any chemical substance. This can be evaluated based on physical, physiological and histological evidence. Physiological evidence is more complex in the present set up as it involves study of complex tissue processes and chemical elements. The physical and histological changes are rather easy as it only composes evaluation of rabbit weight in response to treatment, surface area of aortic intima lesion and evident histological changes on the endothelium of aorta. Any evidence of deviation of named parameters might simply indicate histo-inhibitory processes.

It was observed that the mean area fraction of aortic intima in vehicle control group significantly increased as compared to negative control group (p=0.0001). These findings are similar to observations of (Centa *et al.*, 2019)when quantifying the atherosclerotic lesion on mice. The researcher noted that aortic arch was more prone to development of lesions and lesions alone was not adequate enough to cause harm unless its components were evaluated. In the present study, the rabbits were exposed to high fat diet of 50mg for 7 weeks and it was observed that lesions developed within the aortic intima. It was observed that the mean area fraction of the lesion was 0.51622mm^2. On exposure to a high fat diet atherosclerosis is highly likely to occur. It is usually characterized by damage to endothelial cells of aortic tunica intima which leads to accumulation of LDL. Such damage increases permeability of arterial wall of aorta which increases accumulation of LDL in these vessels (Alfarisi *et al.*, 2020) .

Damaged endothelial cells adhere to nearby white blood cells causing morphological changes, release of free radicals that interact with LDL causing oxidation (Alfarisi *et al*., 2020) This leads to increased oxidative stress, inflammation and damage to endothelium causing endothelial dysfunction (Lian *et al*., 2018). Endothelial dysfunction and chronic inflammation contribute to formation, growth and rapture of atherosclerotic plaque which cause coronary artery heart attack (Marchio *et al*., 2019). Therefore, the present study postulates that presence of high fat diet might have caused endothelial damage, caused permeation of LDL into aorta and interacted with white blood cells causing oxidation that led to formation and growth of atherosclerosis lesion.

It was observed that the mean area fraction of *W. ugandensis* histo-inhibitory group significantly (P=0.0001) reduced as compared to vehicle control group that was subjected to high fatty diet. This observation is similar to that of a previous study (Mandlik & Namdeo, 2021) which found out that *Ashwagandha Somnifera* demonstrated a protective effect against middle cerebral artery occlusion. The researcher argues that this was achieved as the active compounds of the plant have ability to reduce lesion area by reducing the oxidative stress induced during inflammation process and ensuring that there is balance of anti-inflammatory mediators. This plant has been adopted as the two are from a similar family therefore may have common phytochemical compounds. Similar results were observed in a study of (Omara *et al*., 2022) on *Clausena anisata* in ethanolic and aqueous extracts that significantly reduced atherosclerotic plaque. According to (Kimondo, 2020), who made the same observations about a Maasai plant called ilkisonko that had a high phenolic content and, as a result, had beneficial antioxidant and anti-inflammatory properties. This study therefore, attributes the significant reduction of area fraction of atherosclerotic lesion to high levels of flavonoids, phenol and Phyto-steroids that were present on phytochemical analysis.

On histological observation, it was observed that the negative control group had a normal tunica intima with well disturbed endothelial cells covering endothelial layer of thin connective tissue. The endothelium had a simple squamous epithelial lining which concurs with the findings of (Milutinović *et al*., 2020). On vehicle control, the tunica intima appeared to have increased in size, endothelial cells and nucleus sparsely distributed and had a lipid core lesion that was capped with a thin fibrous cap which makes the lesion unstable. This observation is similar to (Shibata *et al*., 2017) who noted that atherosclerotic lesion in tunica intima undergoes several complex stages including; early fatty streak, fibroatheroma and atheroma which invades tunica intima. This study notes that the obvious changes on tunica intima and development of lesion might have undergone a similar developmental process before inscribing within the tunica intima.

On histo-inhibitory, it was observed that there were fatty streaks evidenced by white spicules which did not stain in the tunica intima in comparison between Atorvastatin and W. *Ugandensis* groups. There were few whitish spicules in histo-inhibitory with Atorvastatin as compared to *W. ugandensis* however, there was no development of atherosclerotic lesion but fatty streak. This could be due to the fact that atorvastatin up regulates the production of nitric oxide which is a significant vasodilator with both anti-inflammatory and antioxidative effects (Mathew, 2014,Sorrentino, 2011) This increases vascular resistance, reduces vascular inflammation and increases antioxidant levels which play a critical role in reducing atherogenesis (Liberale *et al*., 2020). On the other hand, *W. ugandensis,* presence of high levels of flavonoids and tannins which are secondary metabolites

with both antioxidant and anti-inflammatory properties might have played a critical role in preventing development of atherosclerotic lesion. This was achieved due to the physiological and histological activities of reducing oxidation of low lipoproteins thereby reducing atherosclerotic lesions (Ciumărnean *et al*., 2020).

CONCLUSION

W. ugandensis has positive histo-inhibitory effects characterized by failure of formation of atherosclerotic lesion. Use of this plant or its phytochemicals would be of great benefit to the population suffering atherosclerosis as the plant is readily available in the local markets. This can also be greatly supported by its ability to high reduce the area fraction of the plaque within the aortic intima.

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