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Effect of *Stachys mialhesi* de Noé on the inflammation induced by hyperhomocysteinemia in cardiovascular diseases

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ABSTRACT

Homocysteine may mediate long-term oxidative damage at the vascular interface through the generation of potent reactive oxygen intermediates. In this study, we evaluated the effect of the anti-inflammatory and antioxidant Stachys mialhesi extract on the inflammation induced by hyperhomocysteinemia and damages in the aorta, heart and liver of mice. Adult male Mus Musculus mice were systematically divided into four groups of similar mean body weights and fed for 21 days with control and experimental diets. The control group (F) was fed with white bread (0.50mg/mice), group (M) was fed with L-methionine (200mg/kg/day), group (MP) was fed with L-methionine (200mg/kg/day) plus S. mialhesi extract (50mg/kg), and the positive control group (P) was treated with S. mialhesi extract (50mg/kg/day). The experimental diets were given in white bread (0.50mg/mice). The plasma hs-CRP concentrations were elevated significantly after the administration of methionine in high doses to mice. This was associated with the loss and degeneration of endothelium, fenestration and formation of foam cells in the media of aorta, also the alteration of the cardiac muscle and liver necrosis. This is due to the angiotoxic action of homocysteine directed to the aorta, and its toxicity on the heart and liver. These changes were not observed in mice treated with methionine plus the antioxidant and anti-inflammatory S. mialhesi extract. Homocysteine initiated inflammation and mediated early atherogenesis lesions through increased oxidant stress, and the treatment with S. mialhesi extract prevented the endothelial and heart alteration. So, combined to vitamin therapy, natural phytochemicals are sources for natural antioxidants and could be used to protect against the homocysteine mediated free oxygen radicals damages.

Key words: Methionine , Homocysteine, atherogenesis, inflammation, hs-CRP, *Stachys mialhesi.*

INTRODUCTION

Elevated levels of plasma homocysteine have been associated with cardiovascular risk in multiple large-scale epidemiological studies [1] and have been recognized as a strong, independent, and causal risk factor for atherosclerosis [2, 3]. Studies from *in vitro* and *in vivo* investigation have suggested that generation of potent reactive oxygen intermediates, such as superoxide anion radical, hydrogen peroxide and impaired production of endothelial nitric oxide, are central mechanisms by which vascular exposure to elevated levels of homocysteine may mediate long-term oxidative damage at the vascular interface [4, 5]. Theses reactive oxygen intermediates may be a target for therapy development and their inhibition might be a strategy in preventing cardiovascular diseases. Single CRP measurement is one of the predictors of cardiovascular events [6] and CRP was established as a sensitive marker of inflammation a long time ago [7]. Accumulating evidence from various epidemiological prospective studies over the recent years indicates that CRP is an important marker of future cardiovascular risk [8-12]. CRP deposition in human atherosclerotic lesions is now well established [13-15]. The molecule is already detectable in the arterial intima in the earliest stages of atherogenesis and accumulates with lesion progression. In this study, and

following the oral administration of methionine in high doses (200 mg/kg during 21 days to mice), our objective was to measure the plasma hs-CRP concentration in order to determine the effect of homocysteine on vascular inflammation and atherogenesis, as CRP has been identified as a powerful cardiovascular risk marker [16, 17], and may also be causally involved in atherogenesis [18- 21]. In parallel, we examined the aortic, heart and liver histology of mice to confirm the angiotoxic and toxic action of homocysteine. Treatment of hyperhomocysteinemia is primarily through vitamin supplementation; folic acid, and vitamins B6 and B12 are the mainstay of therapy. Betaine and 5-methyl tetrahydro-folate are also effective in lowering homocysteine levels [22]. [22] reported that atherogenesis is promoted through increased oxidant stress by elevated levels of plasma homocysteine, and [23] demonstrated *in vivo* that vitamin therapy in rats has reduced homocysteine plasma levels. In this work, a continuation of our previous works on Lamiaceae [24-34], we also substituted vitamin therapy by phytotherapy. Our objective was to evaluate the protective and preventive effect of *S. mialhesi* extract via its antioxidant and anti-inflammatory activity.

MATERIALS AND METHODS

Animals and Diets

Adult male *Mus Musculus* mice (2.5-3 months old), weighing (26g–36g) from central pharmacy Algeria were used in this work. They were systematically divided into four groups of similar mean body weights and fed for 21 days with control and experimental diets. The control group (F) was fed with white bread (0.50mg/mice), group (M) was fed with L-methionine (200mg/kg/day), group (MP) was fed with L-methionine (200mg/kg/day) plus *S. mialhesi* extract (50mg/kg/day), and the positive control group (P) was treated with *S. mialhesi* extract (50mg/kg/day). The experimental diets were given in white bread (0.50mg/mice). The mice were housed 5 per cage and kept under standard laboratory conditions of humidity, temperature (25± 1°C) and light (12 h day: 12 h night), and allowed free access to food and water.

Blood Biochemistry

At the end of the study, mice were fasted overnight, and the blood samples were collected from the retro orbital vein into EDTA tubes by using glass capillaries. They were centrifuged immediately, and the plasma was stored at -30°C. The plasma hs-CRP values were measured by the immunoturbidimetric method on a Cobas integra 400 plus analyzer (Roche).

Histology

After the blood samples collection**,** the animals were sacrificed, and samples for light microscopic investigations were obtained from aorta, heart and liver. For histological investigations the aorta was divided into 4 sections (arch, thoracic, abdominal, and iliac). Paraffin slices, 5um thick were stained following the heamatoxylin eosin staining method.

Statistical Analyses

Results were analyzed for differences between the groups across dietary treatments by one –way ANOVA test and Tukey's multiple comparison tests (SPSS version 9).

RESULTS AND DISCUSSION

In this study, we have taken only 3 values from each group because some are outliers.

The present data showed that there is a significant difference in the means for the plasma hs-CRP concentrations between groups P=0,010. The figure 1 showed that the plasma hs-CRP concentration in group (M) (0,493l±0,109) increased significantly when compared to the control group (F) (0,153±0,006) P= 0,015, and the positive control group(P) (0,16±0,005) and P=0,017. Also, the hs-CRP concentration in the group (MP) (0.20±0,042) decreased significantly P=0,034 when compared to group (M), but didn't reach the CRP concentration in the control group (F).

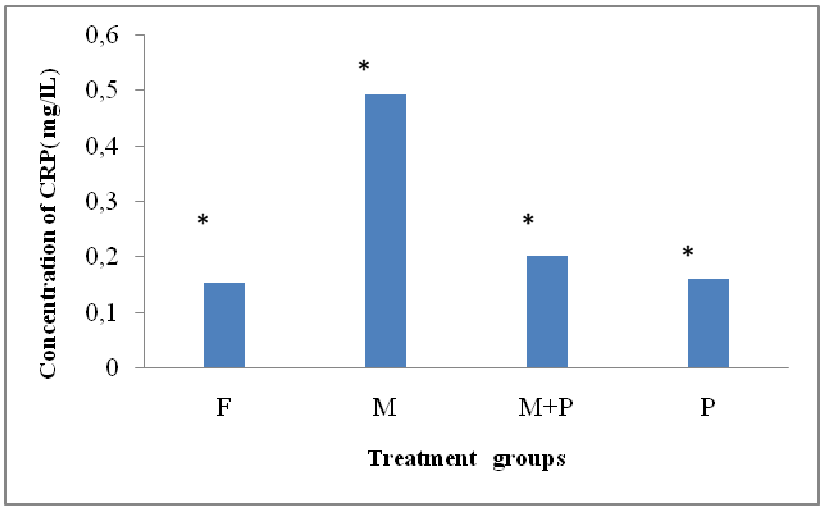

Figure 1: The interaction of L-methionine and *S.mialhesi* extract on the plasma ultra sensitive CRP in mice during 21days application.

In the group (M), which had been fed with 200mg/kg methionine, the aortic intima showed degeneration and desquamation of endothelial cells, we also observed in the media lysis, fenestration, formation of foam cells and oval nuclei **(Figures 2, 3, 8, 9, 13, 14, 19, 20, 21)**.

However, in the control group (F), the aortic sections have intact endothelium and spindle shaped mediocytes nuclei **(Figures 4, 5, 10, 15, 16, 22, 23)**. These results were also observed in the positive control groups (P) **(Figures 7, 12, 18, 26, 27).**

We also observed in group (M), lysis in the histological section of the cardiac muscle and hepatic necrosis in the liver histology **(Figures 28, 32, 33),** contrary to the group (F) and (P) where the heart and liver histology were intact **(Figures 29, 34, 35)** and **(Figures 31, 38**) respectively.

However, the aortic intima of the group (MP) that had been fed with 200mg methionine plus *S.mialhesi* extract showed intact endothelium, and spindle-shaped mediocytes with the absence of foam cells formation. Nevertheless, oval-shaped mediocyte nuclei were observed **(Figures 6**, **11, 17, 24, 25).** In the histological sections of the cardiac muscle and liver**,** no lysis or necrosis was observed **(Figures 30, 36, 37).**

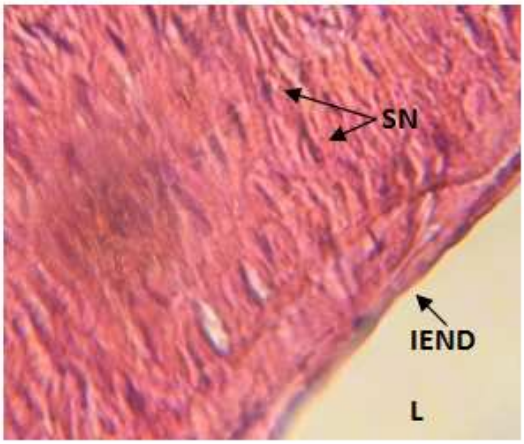

Figure 2 : Histological section of arch aorta, 21 days of oral methionine application (200 mg/kg/day), Hematoxylin eosin staining (x100)

ENS: Endolysis, F: Fenestration

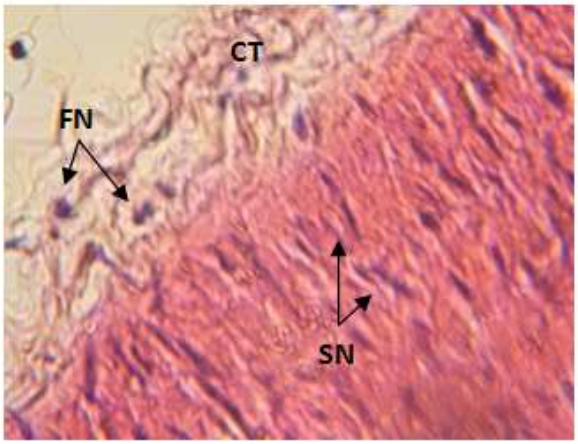

Figure 3: Histological section of arch aorta, 21 days of oral methionine application (200 mg/kg/day), Hematoxylin eosin staining (x1000).

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FC: Foam cells, ON: Oval nuclei, ENS: Endolysis, EF: Elastic fiber

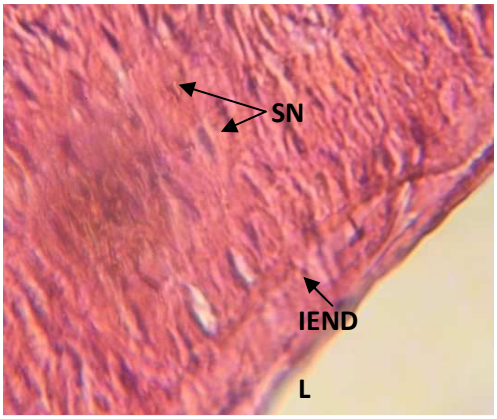

Figure 4: Histological section of arch aorta, control's , Hematoxylin eosin staining (x400).

IEND: Intact Endothelium, SN: Spindle nucleic , L: Lumen

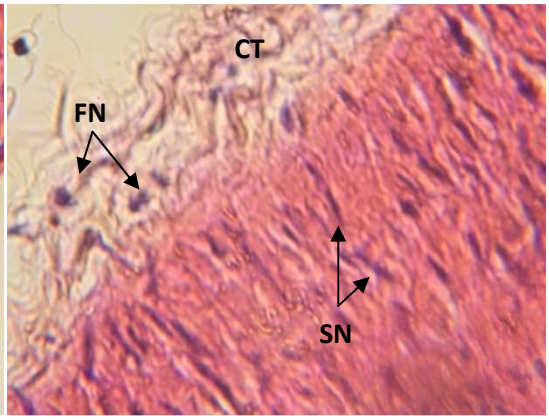

Figure 5: Histological section of arch aorta, control's , Hematoxylin eosin staining (x400).

SN: Spindle .nucleic, CT: connective tissue, FN: Fibroblast nuclei

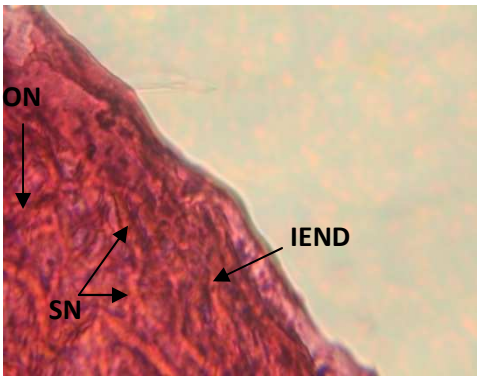

Figure 6: Histological section of arch aorta, oral methionine (200 mg/kg/day) + S.mialhesi extract (50mg/kg/day) application , Hematoxylin eosin staining (x400).

SN: Spindle nucleic, IEND: Intact Endothelium, ON: Oval nuclei.

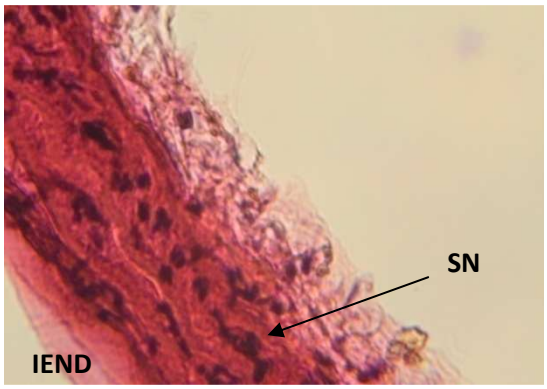

Figure 7: Histological section of arch aorta, positive control's Hematoxylin eosin staining (x400).

IEND: Intact Endothelium , SN: Spindle nucleic

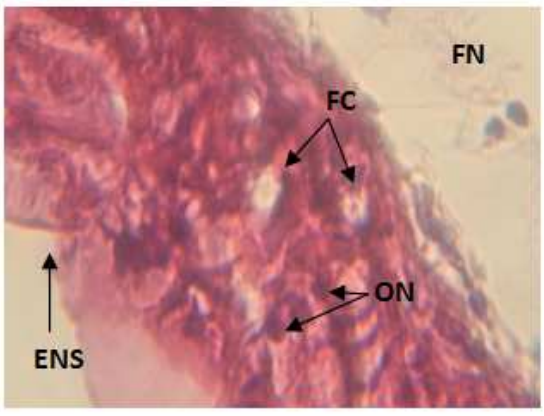

Figure 8: Histological section of thoracic aorta, 21 days of oral methionine application (200 mg/kg/day), Hematoxylin eosin staining (x400).
FC: Foam cells, ON: Oval nuclei, ENS: Endolysis, FN: Fibroblast nuclei

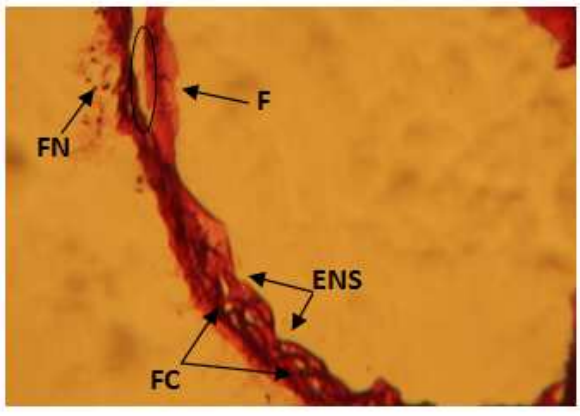

Figure 9 Histological section of thoracic aorta, 21 days of oral methionine application (200 mg/kg/day), Hematoxylin eosin staining (x100).
FC: Foam cells, ENS: Endolysis, FN: Fibroblast nuclei, F: Fenestration

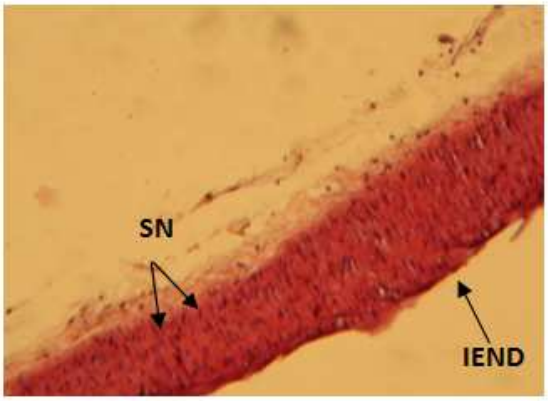

Figure 10: Histological section of thoracic aorta, Control's Hematoxylin eosin staining (x100). IEND: Intact Endothelium, SN: Spindle nucleic

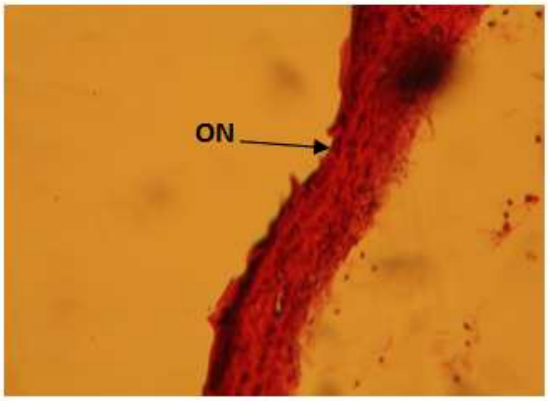

Figure 11: Histological section of thoracic aorta, oral methionine (200 mg/kg/day) + S.mialhesi extract (50mg/kg/day) application , Hematoxylin eosin staining (x100). ON: Oval nuclei

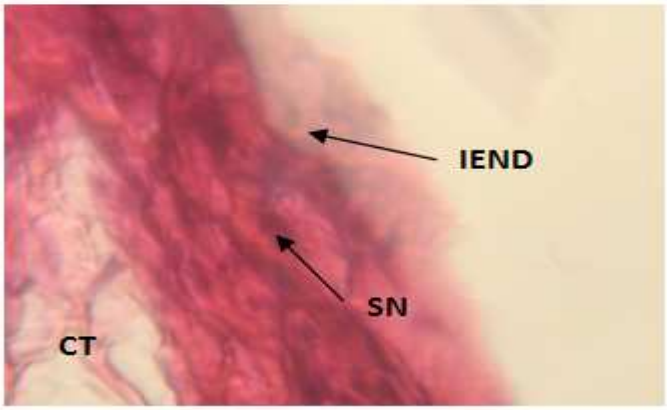

Figure 12 Histological section of thoracic aorta, positive control's Hematoxylin eosin staining (x400). SN: Spindle nucleic , IEND: Intact Endothelium CT: connective tissue,

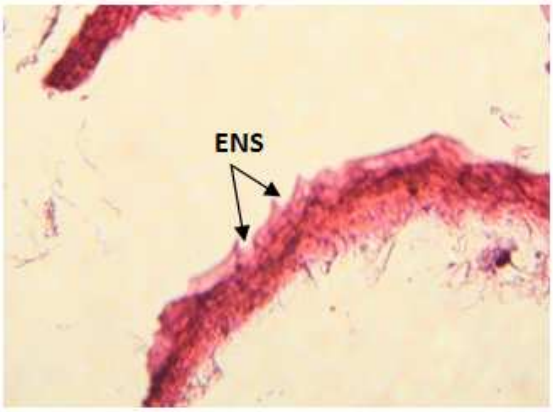

Figure 13: Histological section of the abdominal aorta, 21 days of oral methionine application (200 mg/kg/day), Hematoxylin eosin staining (x100.)

ENS: Endolysis,

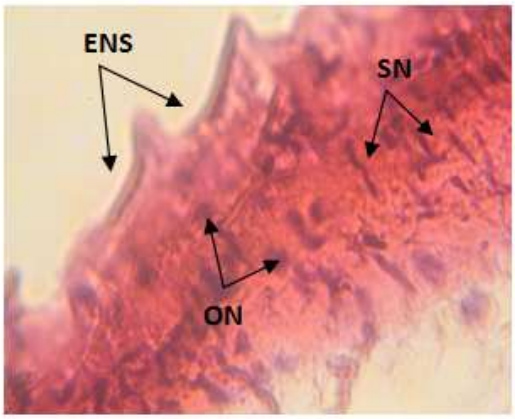

Figure 14: Histological section of the abdominal aorta, 21 days of oral methionine application (200 mg/kg/day), Hematoxylin eosin staining (x400).

SN: Spindle nucleic, ON: Oval nucleic, ENS: Endolysis,

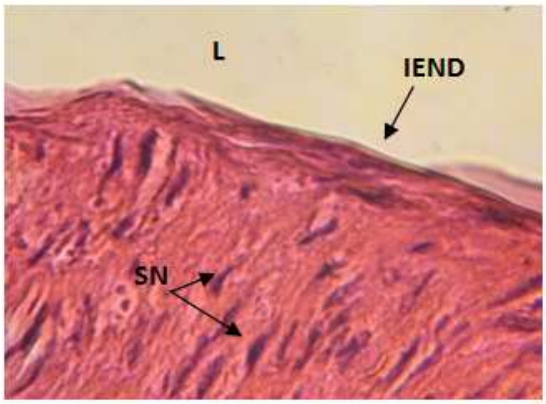

Figure 15: Histological section of the abdominal aorta, control's Hematoxylin eosin staining (x400).
SN: Spindle nucleic, IEND: Intact Endothelium, L: Lumen

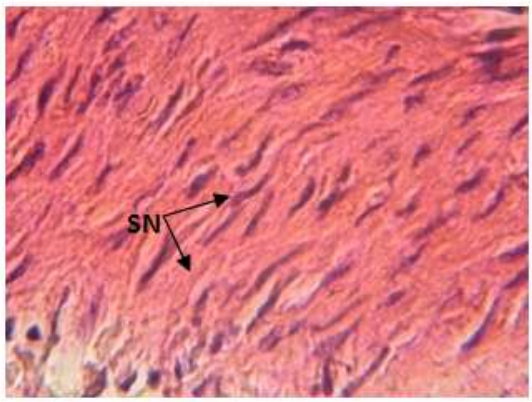

Figure 16: Histological section of the abdominal aorta, control's Hematoxylin eosin staining (x400).

SN: Spindle nucleic

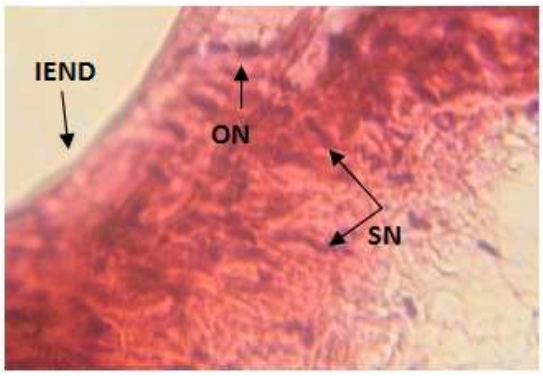

Figure 17: Histological section of the abdominal aorta, oral methionine (200 mg/kg/day) + S.mialhesi extract (50mg/kg/day) application , Hematoxylin eosin staining (x400).

SN: Spindle nucleic, ON: Spindle nucleic, IEND: Intact Endothelium

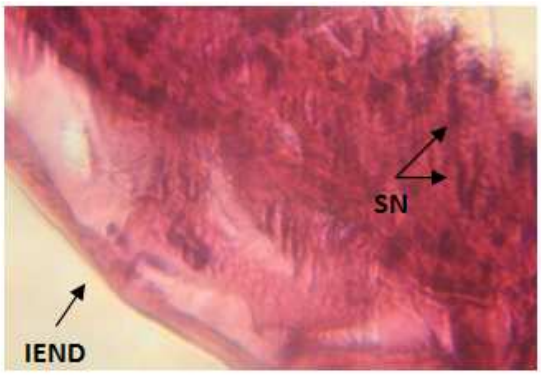

Figure 18: Histological section of the abdominal aorta, Positive control's Hematoxylin eosin staining (x400).

SN: Spindle nucleic, IEND: Intact Endothelium

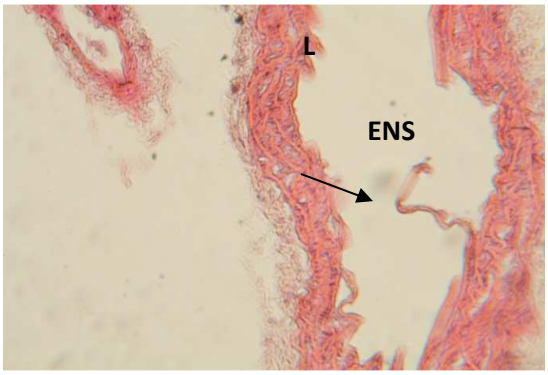

Figure 19: Histological section of iliac aorta, oral methionine (200 mg/kg/day) application Hematoxylin eosin (x100).

ENS:Endolysis L: Lumen

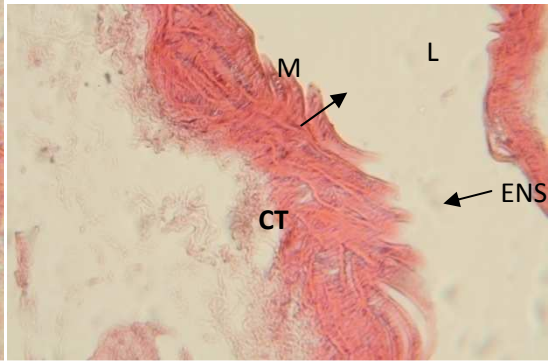

Figure 20: Histological section of iliac aorta, oral methionine (200 mg/kg/day) application Heamatoxylin eosin (x100).

ENS: Endolysis, L: Lumen, M: Migration of muscular cells CT : Connective tissue

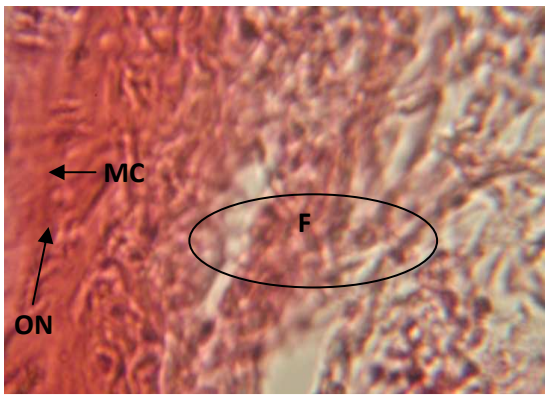

Figure 21: Histological section of iliac aorta, oral methionine (200 mg/kg/day) application Heamatoxylin eosin (x400).

MC : Muscular cells, ON : oval nuclei F: Fibroblast

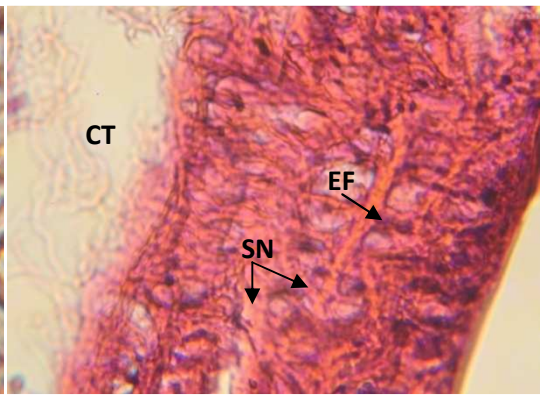

Figure 22: Histological section of iliac aorta, control's Hematoxylin eosin staining (x400).

SN: Spindle nucleic, EF: Elastic fiber, CT: connective tissue,

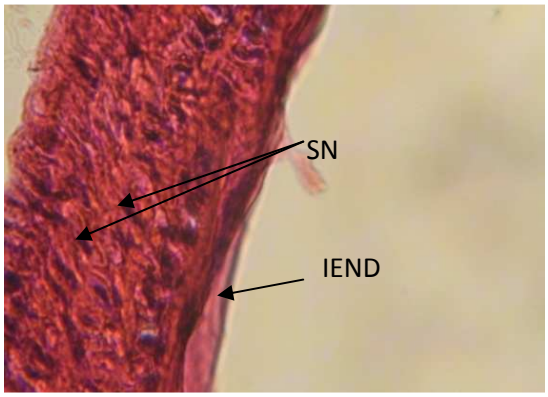

Figure 23: Histological section of iliac aorta, control's Hematoxylin eosin staining (x400).

SN: Spindle nucleic, IEND: Intact Endothelium

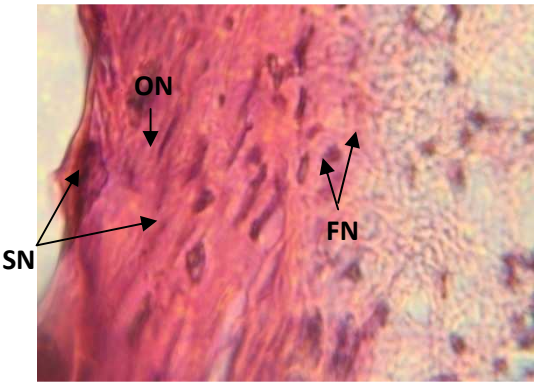

Figure 24: Histological section of iliac aorta, oral methionine (200 mg/kg/day) + S.mialhesi extract (50mg/kg/day) application Hematoxylin eosin staining (x400).

SN: Spindle nucleic, ON: Oval nuclei, FN: Fibroblast nuclei

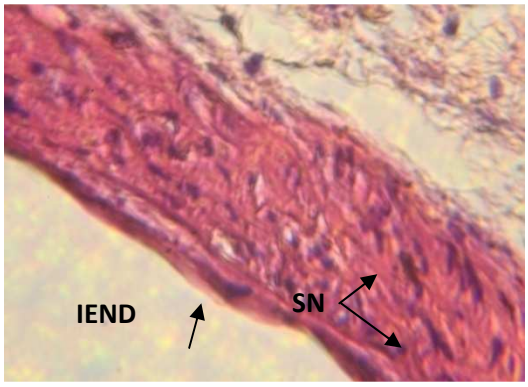

Figure 25: Histological section of iliac aorta, oral methionine (200 mg/kg/day) + S.mialhesi extract (50mg/kg/day) application Hematoxylin eosin staining (x400).

SN: Spindle nucleic, IEND: Intact Endothelium, ON: Oval nuclei

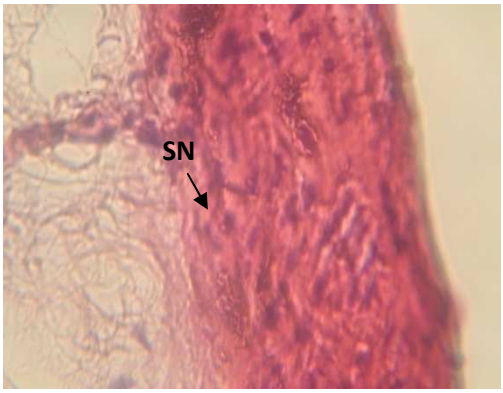

Figure 26: Histological section of the iliac aorta, positive control's Hematoxylin eosin staining (x400).

SN: Spindle nucleic, CT: connective tissue,

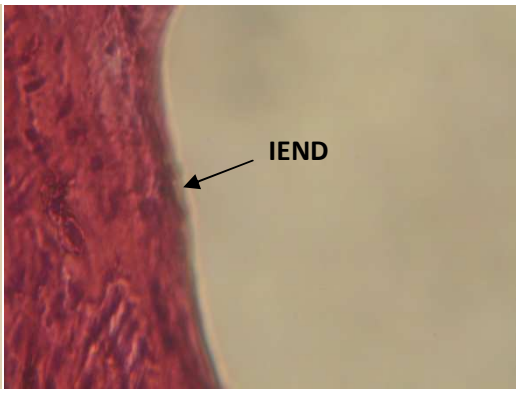

Figure 27: Histological section of the Iliac

aorta, positive control's Hematoxylin eosin staining (x400).

IEND: Intact Endothelium

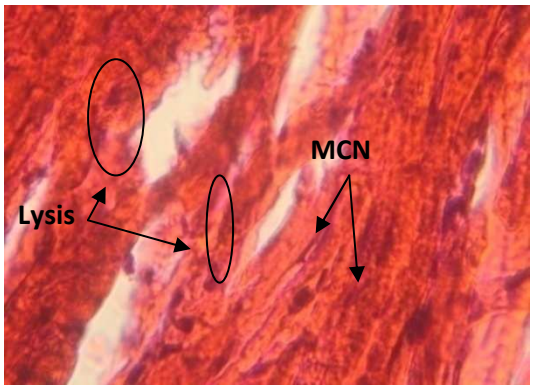

Figure 28: Histological section of the cardiac muscle. 21 days of oral methionine application (200 mg/kg/day), Hematoxylin eosin staining (x400)

MCN: muscle cell nuclei, Lysis.

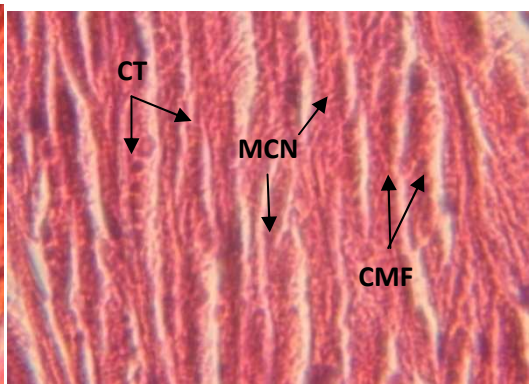

Figure 29: Histological section of the cardiac muscle. Control's Hematoxylin eosin staining (x400).

MCN: muscle cell nuclei, CMF: Cardiac muscle fibers, CT: Connective tissue.

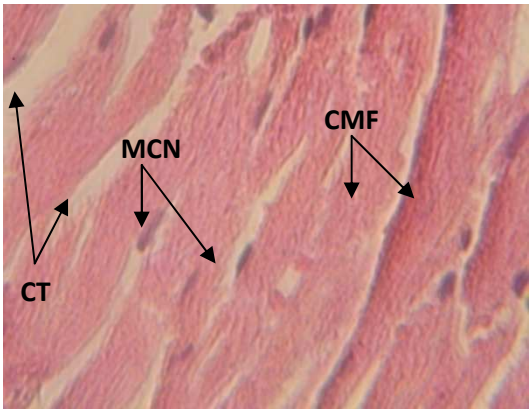

Figure 30: Histological section of the cardiac muscle. 21 days of oral methionine (200 mg/kg/day) + S.mialhesi extract (50mg/kg/day) application Hematoxylin eosin staining (x400). MCN: muscle cel nuclei, CMF: Cardiac muscle fibers, CT: Connective tissue.

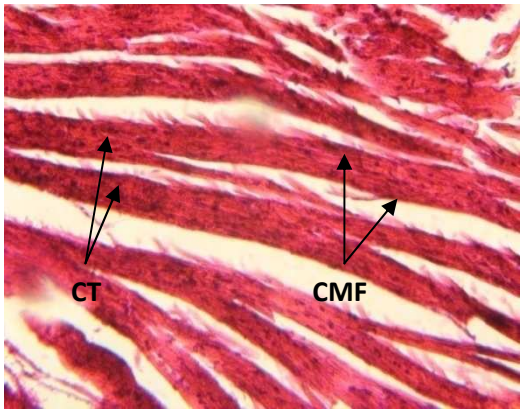

Figure 31: Histological section of the cardiac muscle. positive Control's Hematoxylin eosin staining (x100).

CMF: Cardiac muscle fibers, CT: Connective tissue.

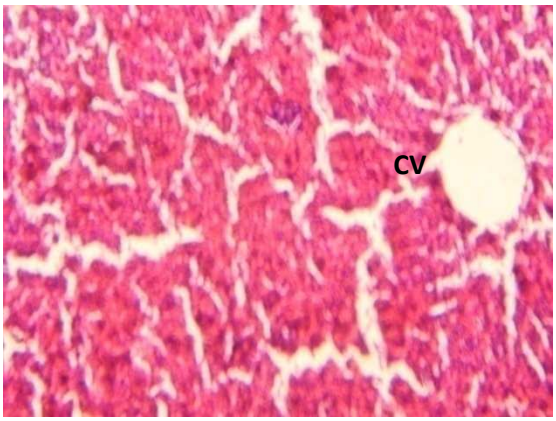

Figure 32: Histological section of the liver, 21 days of oral methionine application (200 mg/kg/day), Hematoxylin eosin staining (x100).

CV: Centro lobular vein

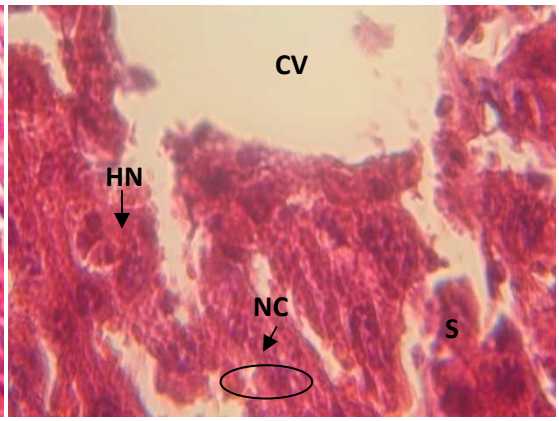

Figure 33: Histological section of the liver, 21 days of oral methionine application (200 mg/kg/day), Hematoxylin eosin staining (x400).

S: Sinusoid, HN: Hepatocyte nuclei, CV: Centro lobular vein, NC: Necrosis

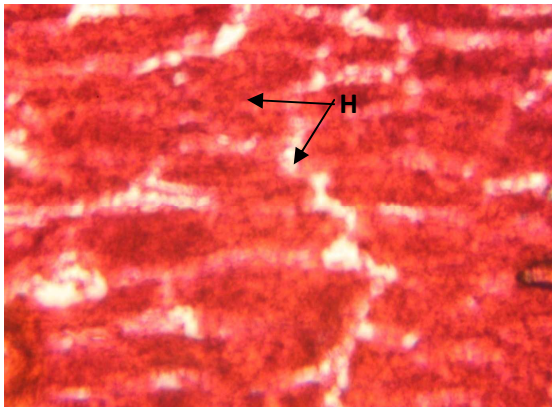

Figure 34: Histological section of the liver, control's Hematoxylin eosin staining (x100).

H: Hepatocytes

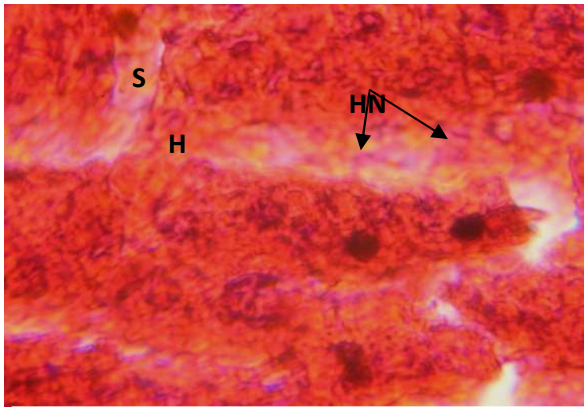

Figure 35: Histological section of the liver, control's Hematoxylin eosin staining (x400).

S: Sinusoid, HN: Hepatocyte nuclei, H: Hepatocyte

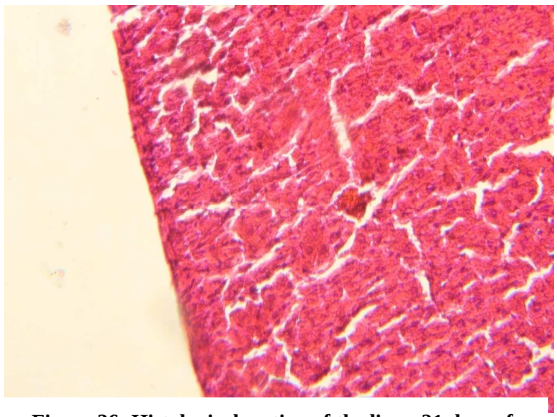

Figure 36: Histological section of the liver, 21 days of oral methionine (200 mg/kg/day) + S.mialhesi extract (50mg/kg/day) application Hematoxylin eosin staining (x100).

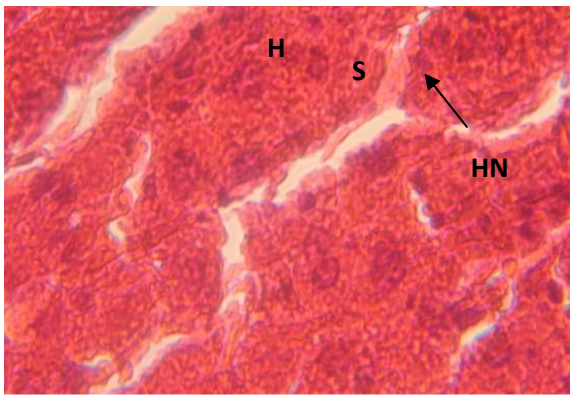

Figure 37: Histological section of the liver, 21 days of oral methionine (200 mg/kg/day) + S.mialhesi extract (50mg/kg/day) application Hematoxylin eosin staining (x400).
S: Sinusoid, HN: Hepatocyte nuclei, H: Hepatocyte

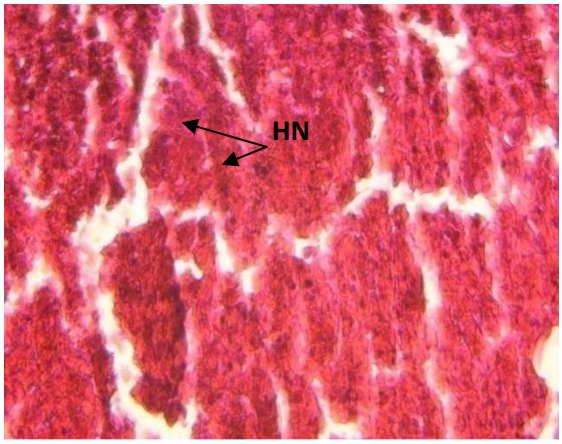

Figure 38: Histological section of the liver, positive control's Hematoxylin eosin (x400).

HN: Hepatocyte nuclei

The group (M) showed a significant increase in the level of plasma hs-CRP compared to the control group. This result could reflect the initiation of an inflammatory process confirmed by the histological investigation of the aorta. In the group (PM) we noticed that *S.mialhesi* extract lowered the plasma hs-CRP level because of its confirmed anti-inflammatory effect, and this due to the presence of established polyphenolic compounds such as tannin and flavonoids [34]. However, the positive control group (P) revealed a higher plasma hs-CRP level than the control group (F) both in a non significant way. This difference is interpreted by the fact that the *S. mialhesi* compounds could possess anti and pro-inflammatory properties [35] .

The oral administration of methionine exerted an angiotoxic activity on the aorta and a toxic effect on the heart and liver. This was observed through the loss and degeneration of endothelium, formation of foam cells in the different sections of the aorta, alteration of the cardiac muscle, and liver necrosis.

Deity excess methionine caused a toxic effect on liver, kidney and heart tissues. [36] and our results are in agreement with the previous experimental studies of [23]. However, the aortic alteration was strongly observed in the aortic arch in contrast to the previous study [23] which was mostly observed in the iliac aorta.

When constant frictional forces are imposed on the endothelium, due to the passage of the blood, anti-inflammatory and antioxidant properties are conferred to the endothelium [37]. But at curved and bifurcating arteries impose a physical constraint to the blood flow, which weakens the frictional forces, leads to homodynamic perturbations of the vascular endothelium, and generates endothelial dysfunction [38, 39] .This explains the fact that the atherosclerotic plaque develops preferentially in the curved and bifurcating arteries [39].
Homocysteine can undergo autoxidation in the plasma or intracellularly, to form various reactive oxygen species. The potent reactive superoxide and hydrogen peroxides, which are produced during this process, are mainly responsible for the vascular toxicity of homocysteine via the formation of oxidized low density lipoprotein (ox-LDL). The oxidation of LDL in the artery wall is believed to be the primary event leading to the initiation and progression of atherosclerosis [40] .

Qualitative phytochemical analysis of the *S. mialhesi* extract revealed that it contains natural physiologically active substances such as terpenoids, flavanoids and phenolic compounds, and it was also showed to exhibit significant antioxidant effect in laboratory animals [24].

The vasculo-protective properties of flavonoïds were revealed by the observation of improvements of the endothelial function after the consumption of flavonoïds rich drinks, such as black tea or red wine. These effects were apparently attributed to the neutralization of free radical,s and pro-inflammatory mediators, but also to the strengthening of the bioactive nitric oxide, an anti-inflammatory mediator and a vasodilator with an antiplatelet concomitant activity [41]. It has been also reported that Polyphenolic substances derived from cocoa powder could contribute to reducing the cholesterol LDL, increasing the cholesterol HDL and limiting the LDL oxidation [42]. In addition, the study of [43] suggested that an increase intake of antioxidants appeared to be protective in cardiovascular diseases.

The macrophages internalize the oxidized LDL [44] and are overloaded with cholesterol. This leads to their transformation to foam cells that accumulate in the arterial wall [45]. These foam cells were not observed in the aorta sections of group (PM), which confirms that homocysteine mediated early atherogenesis lesions through increased oxidant stress. The treatment with the antioxidant *S.mialhesi* extract prevented the endothelial alteration. *S.mialhesi* extract contains polyphenols that are effective scavengers of free radicals by virtue of hydroxyl groups. This confer them the ability to donate electrons, and thus to quench the free radicals [46].

CONCLUSION

Following the oral administration of methionine in high doses, its degradation product, homocysteine initiated an inflammatory process determined by the elevation of the plasma hs-CRP, and confirmed by an angiotoxic activity on the aorta, and a toxic effect on the heart and liver. This was observed through the loss and degeneration of endothelium, formation of foam cells, the alteration of the cardiac muscle, and hepatic tissue necrosis. Following its auto oxidation, homocysteine induced early atherogenesis lesions by the production of free oxygen radicals. The treatment with the antioxidant and anti-inflammatory *S. mialhesi* extract prevented the endothelial alteration, and the heart and liver damages. The treatment of hyperhomocysteinemia is primarily through vitamin supplementation in order to reduce homocysteine plasma levels, but it could be also combined to an antioxidant treatment obtained from natural phytochemicals, a source for natural antioxidants to protect against the homocysteine mediated free oxygen radicals damages.

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