Extracted *Anaxagorea luzonensis* A. Gray Restored Impairment of Endothelium-Dependent Vasorelaxation Induced by Homocysteine Thiolactone in Rat Aortic Rings

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Objective: *To investigate the beneficial effects of Anaxagorea luzonensis (AL) extract on homocysteine thiolactone (HTL)-induced impairment of endothelium-dependent relaxation in rat aortic rings. The mechanisms involved in the effects of AL on endothelial dysfunctions by HTL are also examined.*

Material and Method: *Aortic rings from male Wistar rats were co-incubated for 90 minutes with L-arginine (3 mM), a precursor of nitric oxide (NO); superoxide dismutase (SOD, 200 U/mL), a scavenger of superoxide anion; indomethacin (10 μM), a cyclooxygenase (COX) inhibitor; SC560 (10 μM), a COX-1 inhibitor; NS398 (10 μM), a COX-2 inhibitor; or SQ29548 (1 μM), a thromboxane A_2 receptor antagonist in the presence of HTL (1 mM). After 90 minutes of incubation period, the rings were pre-contracted with methoxamine, and then carbachol was cumulatively added to the bath. AL (1 and 3 μg/mL) was co-incubated with 1 mM HTL in the presence of N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM), a NO synthase inhibitor, and p-hydroxymercurybenzoate (PHMB, 10 μM), a sulfhydryl group blocking agent. Changes in tension were measured using an isometric force transducer and recorded on the PowerLab.*

Results: *Endothelium-dependent vasorelaxation to carbachol was impaired after exposure of aortic rings to HTL (0.3 and 1 mM). The inhibitory effects of HTL (1 mM) on relaxant responses to carbachol were restored by L-arginine, SOD, indomethacin, SC560 and SQ29548, but not NS398. Interestingly, AL reduced impairment of vasorelaxation induced by HTL (1 mM). However, L-NAME and PHMB largely inhibited the protective effects of AL.*

Conclusion: *These results suggest that HTL-induced impairment of endothelium-dependent vasorelaxation may occur via decreased NO release, and generation of oxygen free radical. This study first shows that enhancement of TxA_2 production via COX-1 pathway is involved in HTL-induced endothelial dysfunctions. The protective effects of AL on impairment of relaxation by HTL may be related to increasing NO production and sulfhydryl-dependent.*

Keywords: *Anaxagorea luzonensis, Homocysteine thiolactone, Thromboxane A_2, Oxygen free radicals, Nitric oxide*

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Anaxagorea luzonensis A. Gray (AL) belongs to the Annonacea family. The heartwood of AL has been widely used as a traditional plant in Thailand. It acts as a health promoting herb, blood tonic, antipyretic, and relieves muscle pain[1,2]. However, there is less scientific evidence for pharmacological effects of AL extract. A previous report has shown that AL extract, rich in flavones, flavonones, and flavonols, has an estrogenic activity[3]. Moreover, xanthones and flavonoids, isolated from the bark of AL, exert an antioxidant activity[2]. Recently, a study using rat aortic rings has shown that AL extract induces vasorelaxation via endothelium-derived nitric oxide, activation of K^+ channels, and inhibition of Ca^{2+} influx[4].

Elevated plasma level of homocysteine thiolactone (HTL), a homocysteine metabolite, is a risk factor for cardiovascular diseases. It promotes atherosclerosis, thrombosis, and platelet aggregation[5-7]. Concerning deleterious effects on cardiovascular functions, HTL decreases systolic left

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ventricular pressure, heart rate, and coronary flow in the isolated rat heart[8,9]. In addition, HTL impairs endothelium-dependent vasorelaxation in isolated rat aortae[10,11], and animals treated with HTL[12]. Endothelial dysfunctions induced by HTL are mediated by decreasing nitric oxide release from the endothelium and by enhancing the production of reactive oxygen species[6,10,11]. Moreover, the vascular responses to HTL are more sensitive in adult rats than young rats[13]. In the present study, we sought to examine whether AL extract might exerts beneficial effects on HTL-induced impairment of endothelium-dependent relaxation in isolated rat aortic rings. We also investigated the mechanisms involved in the effects of AL on HTL-induced endothelial dysfunctions.

Material and Method

Extraction of Anaxagorea luzonensis A. Gray (AL)

The dichloromethane extract used in this study was extracted from the heartwoods of *Anaxagorea luzonensis.* The extraction procedure has been fully described in a previous study[14].

Tissue preparation

Experiments were performed using aortic rings obtained from male Wistar rats (300-350 g) purchased from National Laboratory Animal Center, Mahidol University, Thailand. Rats were housed in standard environmental condition (25°C) under a 12 h light/dark cycle, and fed with standard laboratory rat chow and tap water ad libitum. All experimental procedures were reviewed and approved by the Animal Research Ethics Committee of the Faculty of Medicine, Srinakharinwirot University (15/2555).

Male Wistar rats were anaesthetized with Zoletil 50 mg/kg (tiletamine hypochloride and zolazepam hypochloride) injected into the quadriceps muscle, and killed by cervical dislocation. Following a thoracotomy, the thoracic aorta was carefully removed and cleaned of fat and connective tissue. The aortic segment was cut into 5 mm rings. Each ring was transferred to a jacketed organ bath containing oxygenated (95% O_2/ 5% CO_2) Krebs-Henseleit solution (composition, mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, D-glucose 10) that was maintained at 37°C. The isolated rings were mounted between two triangular stainless steel hooks passed through the ring lumen, stretched to an optimal passive tension of about 1 g, and maintained at this tension for 1 h. The upper hook was connected to an isometric force transducer, and changes in isometric force were recorded on a PowerLab recording system.

Experimental protocol

Following an 1-hour equilibration period, aortic rings of control group were incubated with vehicle (distilled water), and the rings of HTL group were incubated with HTL (0.3 and 1 mM) for 90 minutes. After 90 minutes of incubation period, methoxamine, an alpha adrenoceptor agonist, was used to increase the tone by approximately 1 g. Once a stable tone was achieved, concentration-response curves of carbachol (1 nM-100 μM), and sodium nitroprusside (0.1 nM-10 μM), were established in the presence and absence of HTL (1 mM).

To investigate the mechanisms involved in the deleterious effects of HTL on vascular functions, the rings were co-incubated for 90 minutes with L-arginine (3 mM), a precursor of nitric oxide (NO); SOD (200 U/mL), a scavenger of superoxide anion; indomethacin (10 μM), a cyclooxygenase (COX) inhibitor; SC560 (10 μM), a COX-1 inhibitor, NS398 (10 μM); a COX-2 inhibitor; or SQ29548 (1 μM), a thromboxane A_2 receptor antagonist in the presence of HTL (1 mM). After 90 minutes of incubation period, the rings were pre-contracted with methoxamine, and then carbachol was cumulatively added to the bath.

To investigate the effects of AL on HTL-induced impairment of endothelium-dependent relaxation, AL (1 and 3 μg/mL) was co-incubated with 1 mM HTL for 90 minutes. In this study, we did not use higher concentration of AL as methoxamine could not induce contraction. Moreover, the mechanisms involved in the beneficial effects of AL were studied by co-incubation with N^G-nitro-L-arginine methyl ester (L-NAME), a NO synthese inhibitor; or p-hydroxymercurybenzoate, a sulfhydryl group blocking agent in the presence of HTL (1 mM) and AL (3 μg/mL) for 90 minutes. After 90 minutes of incubation period, contractions were induced with methoxamine, and then carbachol was cumulatively added to the bath.

Statistical analysis

The concentration of vasorelaxant giving half-maximal relaxation (EC_{50}) and maximal responses (R_{max}) were obtained from the concentration-response curve fitted to a sigmoidal logistic equation using the Graph Pad Prism package[14]. Difference between groups were tested for statistical significance by analysis of variance followed by Bonferroni's post-hoc test. Results were expressed as mean $\pm$ SEM. $p<0.05$ was considered statistical significance. The number of aortic rings in

each group is represented by n.

Drugs and chemicals

All drugs and chemicals were purchased from Sigma-Aldrich Chemical Company, but zoletil was purchased from Virbac. All drugs were dissolved in the distilled water, except for AL, SC560, NS398 (stock solution in dimethyl sulfoxide), SQ 29548 (stock solution in ethanol, and SQ29548 (stock solution in ethanol).

Results

Effects of HTL on vasorelaxation of rat aortic rings

Carbachol induced concentration-dependent vasorelaxation (R_{max} = 106±4% with EC_{50} = 6.12±0.09, n = 6). Endothelium-dependent vasorelaxations to carbachol were significantly (p<0.001) reduced after incubation of aortic rings with HTL (0.3 and 1 mM) (R_{max}: control = 106±4%; 0.3 mM HTL = 83.7±3.5%; 1 mM HTL = 48.3±2.5%, n = 6, Fig. 1). However, endothelium-independent vasorelaxations to sodium nitroprusside were unaffected by HTL (data not shown).

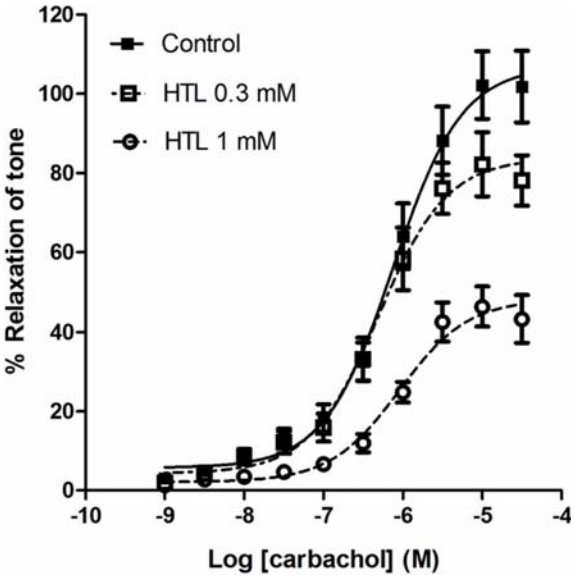

Fig. 1 Effects of pre-treatment with homocysteine thiolactone (0.3 and 1 mM HTL) for 90 minutes on carbachol-induced vasorelaxation in rat aortic rings. Data were shown as mean ± SEM, n = 6.

Effects of drugs on endothelium-dependent vasorelaxation to carbachol in rat aortic rings

The inhibitory effects of HTL on relaxant responses to carbachol are significantly (p<0.001) prevented after treatment of aortic rings with indomethacin (10 μM), SC560 (10 μM), or SQ29548 (1 μM), but not NS398 (10 μM) (R_{max}: indomethacin = 78.1±4.4%; SC560 = 88.3±4.5%; SQ29548 = 90.8±4.9%; NS398 = 51.4±2.5%; n = 6, Fig. 2).

Treatment of aortic rings with L-arginine (3 mM) or SOD (200U/mL) restored HTL-induced impairment of endothelium-dependent vasorelaxation (R_{max}: L-arginine = 111±2%; SOD = 95.4±4.9%; n = 6, Fig. 3).

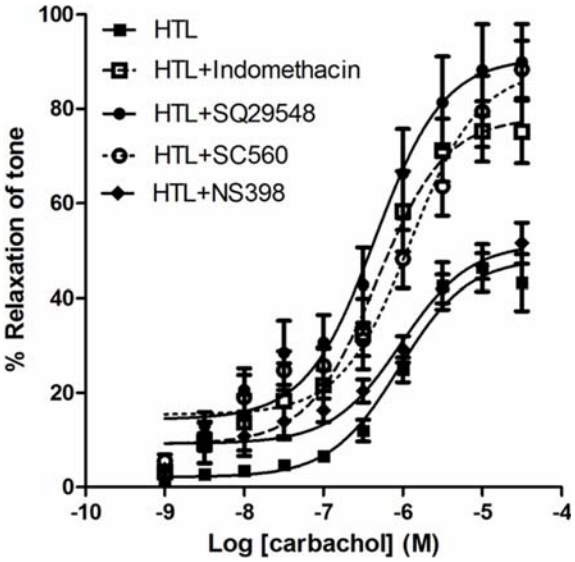

Fig. 2 Effects of pre-treatment with indomethacin (10 μM), SC560 (10 μM), NS398 (10 μM) and SQ29548 (1 μM) in the presence of homocysteine thiolactone (HTL 1 mM) for 90 minutes on carbachol-induced vasorelaxation in rat aortic rings. Data were shown as mean ± SEM, n = 6.

Effects of AL extract and its mechanisms on endothelium-dependent vasorelaxation to carbachol in rat aortic rings

The inhibition of endothelium-dependent vasorelaxation induced by HTL was significantly (p<0.001) attenuated by 3 mg/mL AL (R_{max}: 1 mM HTL = 48.3±2.5%; AL+HTL = 73.6±2.7% n = 6, Fig. 4). In the presence of HTL and AL (3 μg/mL), co-incubation of aortic rings with L-NAME (300 μM) or PHMB (0.05 mM) significantly (p<0.001) attenuated the protective effects of AL on HTL-induced inhibition of endothelium-dependent vasorelaxation (R_{max}: L-NAME = 25.3±2.5%; PHMB = 41.0±2.3%; n = 6, Fig. 5).

Discussion

The present findings in rat aortic rings demonstrated that HTL inhibited endothelium-dependent

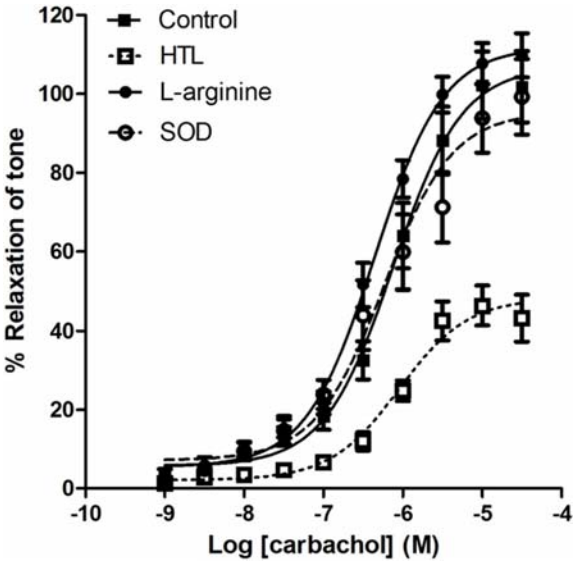

Fig. 3 Effects of pre-treatment with L-arginine (3 mM) and SOD (200 U/mL) in the presence of homocysteine thiolactone (HTL 1 mM) for 90 minutes on carbachol-induced vasorelaxation in rat aortic rings. Data were shown as mean ± SEM, n = 6.

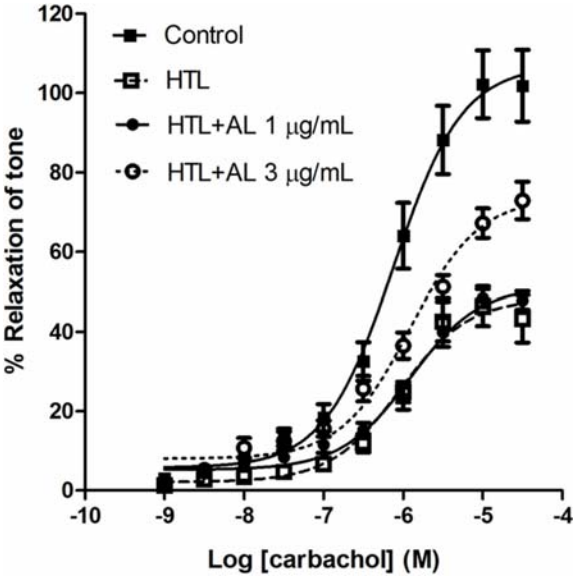

Fig. 4 Effects of AL (1 and 3 μg/mL) in the presence of homocysteine thiolactone (HTL 1 mM) for 90 minutes on carbachol-induced vasorelaxation in rat aortic rings. Data were shown as mean ± SEM, n = 6.

vasorelaxation induced by carbachol in a dose-dependent manner. However, HTL did not affect

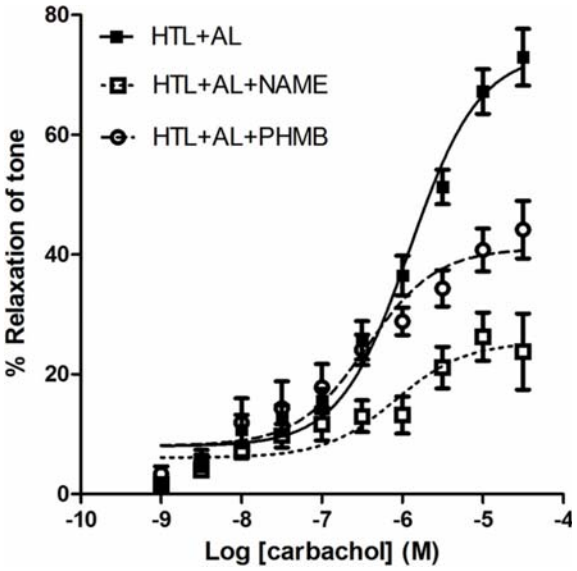

Fig. 5 Effects of N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) or p-hydroxymercurybenzoate (PHMB, 10 μM) in the presence of extracted AL (3 μg/mL) and homocysteine thiolactone (HTL 1 mM) for 90 minutes on carbachol-induced vasorelaxation in rat aortic rings. Data were shown as mean ± SEM, n = 6.

endothelium-independent vasorelaxation to sodium nitroprusside. These results suggest that HTL had no effect on NO-induced endothelium-independent vasorelaxation. We then sought to investigate the mechanisms involved in the inhibitory effects of HTL. It was found that L-arginine could restore inhibition of endothelium-dependent vasorelaxation. These results indicate that HTL may reduce the production of nitric oxide. Indeed, previous studies have shown that HTL decreases nitric oxide production in rat aortae[10] and in human aortic endothelial cells[15].

The precise mechanisms involved in the inhibitory effects of HTL on endothelium-dependent vasorelaxation are not fully understood. We found that the impairment of endothelium-dependent vasorelaxation induced by HTL was restored by treating the aortic rings with indomethacin, a COX inhibitor, SC560, a selective COX-1 inhibitor, and SQ29548, a thromboxane A_2 receptor antagonist. However, NS398, a selective COX-2 inhibitor did not affect the inhibitory effects of HTL. These findings suggest that increased production of thromboxane A_2 via a COX-1 pathway contributes to HTL-induced endothelial dysfunctions. These findings are in agreement with a previous study showing that the

production of thromboxane A_2 is increased in venules and skeletal muscle arterioles from hyperhomocysteinemic rats[16,17].

Impairment of endothelial functions induced by homocysteine and HTL, its reactive metabolite, contributes to increasing production of oxygen free radicals, especially superoxide anion[11,18,19]. In agreement with previous findings, we found that SOD, a scavenger of superoxide anions, restored impairment of endothelium-dependent vasorelaxation induced by HTL.

AL has several pharmacological effects including antioxidant and vasorelaxant[2,4]. In this study, we found that extracted AL restored impairment of endothelium-dependent vasorelaxation induced by HTL. Co-incubation of aortic rings with HTL and L-NAME abolished the protective effects of extracted AL. In addition, the effects of extracted AL on endothelial dysfunction by HTL were reduced by PHMB. These results suggest that extracted AL exerts its protective effects on endothelial dysfunctions induced by HTL largely through enhancement of NO production, and sulfhydryl-dependent.

In conclusion**,** the present study has shown that HTL inhibits endothelium-dependent vasorelaxation in rat aortic rings. The inhibitory effects of HTL are mediated by inhibition of nitric oxide release and increased production of oxygen free radicals and thromboxane A_2 via COX1 pathway. Interestingly, extracted AL could restore impairment of endothelium-dependent vasorelaxation by HTL, which may involve increasing production of nitric oxide and depend on sulfhydryl group. These findings provide pharmacological evidence to support the use of extracted AL to prevent endothelial dysfunctions induced by HTL.

What is already known on this topic ?

Previous studies have reported that extracted AL exerts an antioxidant activity and induces vasorelaxation. Elevated plasma level of HTL, a homocysteine metabolite, causes impairment of endothelial functions. However, there is no evidence for the beneficial effects of AL extract on endothelial dysfunctions induced by HTL.

What this study adds ?

The present study has shown that the protective effects of AL on impairment of vasorelaxation by HTL may be due to increasing NO production and sulfhydryl-dependent. These results first provide pharmacological evidence to support the use of extracted AL to prevent HTL-induced endothelial dysfunctions.

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Potential conflict of interests

None.

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สารสกัด *Anaxagorea luzonensis A. gray* ช่วยลดความเสื่อมในการคลายตัวของหลอดเลือดแบบที่ต้องอาศัยชั้นเยื่อบุหลอดเลือดที่ถูกเหนี่ยวนำโดยสาร *homocysteine thiolactone* ในหลอดเลือดเอออร์ตาของหนูแรท

พัชรินทร์ เทพอารีนันท์, พงษ์พัฒน์ เวชสิทธิ์, พัฒทรา สวัสดี

วัตถุประสงค์: เพื่อศึกษาผลของสารสกัด *Anaxagorea luzonensis (AL)* ต่อความผิดปกติในการคลายตัวของหลอดเลือดแบบที่ต้องอาศัยชั้นเยื่อบุหลอดเลือดที่ถูกเหนี่ยวนำโดยสาร homocysteine thiolactone (HTL) ในหลอดเลือดเอออร์ตาของหนูแรทรวมทั้งกลไกที่เกี่ยวข้อง

วัสดุและวิธีการ: นำหลอดเลือดเอออร์ตาจากหนูแรทเพศผู้แช่ในสารละลาย HTL (1 mM) เป็นเวลา 90 นาที ร่วมกับสารต่อไปนี้ L-arginine (3 mM) ซึ่งเป็นสารตั้งต้นของ nitric oxide (NO); superoxide dismutase (SOD, 200 U/mL) ซึ่งเป็นสารกำจัด superoxide anion; indomethacin (10 μM) ซึ่งเป็นสารยับยั้ง cyclooxygenase (COX); SC560 (10 μM) ซึ่งเป็นสารยับยั้ง COX-1; NS398 (10 μM) ซึ่งเป็นสารยับยั้ง COX-2; หรือ SQ29548 (1 μM) ซึ่งเป็น thromboxane A_2 receptor antagonist หลังจากแช่หลอดเลือด 90 นาที หลอดเลือดถูกทำให้หดตัวด้วย methoxamine และเติม carbachol หลอดเลือดเอออร์ตาของหนูแรทถูกแช่ด้วย AL (1 and 3 μg/mL) และ 1 mM HTL ร่วมกับ N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) ซึ่งเป็นสารยับยั้ง NO synthase และ p-hydroxymercurybenzoate (PHMB, 10 μM) ซึ่งเป็นสารยับยั้ง sulfhydryl group การเปลี่ยนแปลงความตึงตัวของหลอดเลือดวัดด้วย isometric force transducer และใช้ PowerLab บันทึกข้อมูล

ผลการศึกษา: การคลายตัวของหลอดเลือดแบบที่ต้องอาศัยชั้นเยื่อบุหลอดเลือดโดย carbachol ลดลงโดย HTL (0.3 and 1 mM) ซึ่งฤทธิ์ของ HTL (1 mM) ต่อ carbachol ถูกยับยั้งโดย L-arginine, SOD, indomethacin, SC560 and SQ29548 สิ่งที่น่าสนใจคือสารสกัดที่ได้จากต้นกำลังวัวเถลิงลดฤทธิ์ของ HTL (1 mM) อย่างไรก็ตาม L-NAME และ PHMB ยับยั้งฤทธิ์ของ AL

สรุป: ผลการศึกษาแสดงให้เห็นว่า HTL ลดการคลายตัวของหลอดเลือดแบบที่ต้องอาศัยชั้นเยื่อบุหลอดเลือดโดย carbachol ซึ่งอาจเกิดจากการลดลงของการหลั่ง NO และจากการสร้างสารอนุมูลอิสระ การศึกษานี้เป็นการศึกษาแรกที่แสดงให้เห็นว่าการเพิ่มขึ้นของการสร้าง TxA_2 ผ่านทาง COX-1 เกี่ยวข้องกับฤทธิ์ของ HTL ที่เหนี่ยวนำให้เซลล์เยื่อบุหลอดเลือดสร้างสารที่มีผลลดการคลายตัว นอกจากนี้ยังพบว่าสารสกัดที่ได้จากต้นกำลังวัวเถลิงยังมีฤทธิ์ลดการยับยั้งการขยายตัวของหลอดเลือดที่เกิดจาก HTL ซึ่งอาจเกี่ยวข้องกับการเพิ่มการสร้าง NO และเป็นกลไกที่อาศัยหมู่ sulfhydryl