doi.org/10.36721/PJPS.2021.34.2.SUP.781-785.1

Endothelium independent calcium channel blocking pathways mediate antihypertensive effect of *Morus nigra*

Mueen Ahmad Chaudhry[1], Alamgeer[1,2*], Muhammad Naveed Mushtaq[3], Ali Omar Alqarni[4], Bandar Ali Alyami[4], Mater Hussen Mahnashi*[4], Yahya Saeed Alqahtani[4], Muhammad Akram[1], Hafiz Muhammad Irfan[1], Humayun Riaz[5] and Sumera Qasim[6]

[1]Laboratory of Cardiovascular Research and Integrative Pharmacology, College of Pharmacy, University of Sargodha, Pakistan
[2]Punjab University College of Pharmacy, University of the Punjab, Lahore, Pakistan
[3]Department of Pharmacy The University of Lahore, Lahore, Pakistan
[4]Department of Pharmaceutical chemistry, College of Pharmacy, Najran University, Najran, Kingdom of Saudi Arabia
[5]Rashid Latif college of Pharmacy, Lahore, Pakistan
[6]College of Pharmacy, Jouf University, Aljouf, Sakaka, Saudi Arabia

Abstract: Present study was conducted to validate the folkloric claims of *Morus nigra* L. (Moraceae) using invasive blood pressure measuring and *ex vivo* vasorelaxant experimental techniques. Intravenous administration of mn. aq in 0.01-30 mg/kg doses caused significant decrease in mean arterial pressure and heart rate in fructose-induced hypertensive rats. It also showed relaxation in high k^+ [80 mm] and pe (1µm) mediated aortic contraction with ec_{50} 1.25 and 3.72mg/ml values, respectively. Vaso-relaxant effect of mn.aq was partially blocked in presence of l-name with ec_{50}, 5.32mg/ml value, but showed concentration dependent significant inhibition of ligand gated and voltage gated Ca^{+2} channels and intracellular ca^{+2} release, similar to verapamil. Findings of current study designate that aqueous fraction of *M. nigra* possesses antihypertensive activity with concentration-dependent vaso-relaxant effect predominantly mediated through endothelial-independent calcium channel blocking pathways accompanied by partial involvement of endothelium-dependent nos mediated relaxation.

Keywords: Vaso-relaxant, nitric oxide synthase, verapamil, phenylephrine.

INTRODUCTION

Hypertension is the most common disease among cardiovascular disorders and among the leading cause of global morbidities and mortalitie. Antihypertensive drugs considerably reduces the risk of hypertension-related morbidity and mortality (Trial, 2002). It has been reported that about 80% of people in developing countries completely rely on the traditional system of medications for primary health issues (Calvo and Cavero, 2014). Synthetic medicines are not only expensive, but also insufficient to fulfill demand in third world countries (Aslam and Sial, 2014). WHO is shifting its focus towards the rational use of traditional medicine and its incorporation in the list of essential drugs, but it demands an extensive pharmacological study on traditional herbal medicines by using modern techniques (WHO, 2011).

Morus nigra L. belonging to Moraceae family, commonly known as black mulberry (locally: Shahtut). It is found and cultivated extensively all around the world for centuries. *M. nigra* is not only one of the ancient fruit tree, but also has been used in folk practice as diuretic, laxative, antitussive, expectorant, sedative, anxiolytic and hypotensive (Kumar and Chauhan, 2008; Padilha *et al.*, 2010). Current study aimed to validate the folkloric claim of *M. nigra* in hypertension on scientific basis by using invasive and *ex vivo* techniques.

MATERIALS AND METHODS

Chemicals and drugs

Diethyl ether, ethyl acetate, *n*-butanol and *n*-hexan were used for liquid-liquid extraction of crude extract. Acetylcholine, atropine, nitro-L-arginine methyl ester hydrochloride (L-NAME), verapamil, angiotensin-II and phenylephrine hydrochloride (Sigma Chemicals, St. Louis, MO, USA) was used as standard drugs.

Plant materials

M. nigra L. (black mulberry) dried fruits were purchased from herbal market of Lahore. Plant material was identified by a taxonomist of the Department of Botany, GC University, Lahore, Pakistan and specimen voucher was preserved in College of Pharmacy, University of Sargodha.

Crude extract of dried fruits of *M. nigra* was prepared with maceration in 70% aqueous-ethanolic solution, previously described by Chaudhry *et al.* (2021). Liquid-liquid extraction of *M. nigra* crude extract (Mn. Cr) was performed with the aim to collect active constituents in

**Corresponding authors:* e-mail: alam_yuchi@yahoo.com; matermaha@gmail.com

one fraction using solvents of different polarity (*n*-hexan, diethyl ether, ethyl acetate and *n*-butanol, respectively). The crude extract (100g) was liquefied in distilled water and treated with equal volume of organic solvent in separating funnel to prepare their respective fractions. The final residual layer at the end was also vaporized and labeled as aqueous fraction (Alamgeer *et al.*, 2016).

Animals

Male albino rats were obtained from animal house of University of Sargodha and kept under controlled environment. All experiments were performed following ethics guidelines of Animal Ethical Committee (No. IAEC/UOS/2017/11) of University Council (Clark *et al.*, 1997).

Evaluation of antihypertensive effect of aqueous fraction of M. nigra in fructose-induced hypertensive rats

Acute antihypertensive effects of aqueous fraction of *Morus nigra* (Mn. Aq) in fructose fed hypertensive rats (FFHR) was evaluated by using technique described previously by Aslam *et al.* (2016). 10% w/v of fructose solution was given for drinking to the normotensive rats *ad libitum* and blood pressure was estimated invasively at end of six weeks.

Briefly, after the onset of surgical anesthesia intravascular catheterization of the right juggler vein and left carotid artery with polyethylene tubes were performed to administer the increasing doses (0.01-30mg/kg) of test/control drug and to read their effects on blood pressure, respectively. Power Lab equipped with pressure transducer (MLT0699) was used for recording the effects.

Evaluation of vaso-relaxant on the isolated aorta of rats

Vaso-relaxant effect of Mn.Aq was assessed using technique detailed previously (Hu *et al.* 2018). In brief, 3 -4mm aortic rings of albino rats of either sex were suspended in organ baths filled with 10ml Kreb's solution ventilated with carbogen at 37°C temperature. Each aortic ring was equilibrated under 1g resting tension and stabilized with phenylephrine (PE, 1µM). Acetylcholine (1µM) was added in organ bath to check the endothelial integrity. More than 80% relaxation was perceived characteristic of an endothelium intact aorta. Power Lab equipped with isometric force transducer (MLT0201) was used to record the signals.

Determination of endothelial dependent mechanisms

To assess endothelium involvement in vasorelaxant effect, Mn. Aq was administered in cumulative concentrations on PE (1µM) mediated sustained contraction of the aortic ring with intact and denude endothelium. To clarify the mechanism, experiments were conducted further on PE induced aortic contractions pre-incubated for 30 min. with atropine (1 µM): A muscarinic receptor blocker or L-NAME (0.1mM): A NO synthase inhibitor, using technique previously described by Younis *et al.* (2020).

Determination of endothelial independent mechanisms

Inhibition of PE and high K^+ mediated contractions indicates the calcium channel blocking potential either by hindering extra cellular Ca^{+2} influx or by blocking intracellular Ca^{+2} efflux (Younis *et al.*, 2021). A set of experiments were performed to determine the involvement of specific calcium channels blocking mechanism.

Effect on voltage-gated and receptor operated calcium channels

Response of high K^+ was observed in with and without Mn.Aq and control drug (verapamil) by using the technique previously followed by Zhou *et al.* (2017). Any decrease in maximal response of high K^+ was indicator of inhibition of voltage gated calcium channels that was confirmed by calcium blocking activity using the technique followed previously by Qayyum *et al.* (2016).

Phenylephrine concentration response curves (CRC) were constructed using the technique followed previously by Ahmad *et al.* (2020). CRCs were created with increasing concentration of PE (log-2-log 1) in presence and absence of Mn. Aq.

Effect on intracellular calcium stores

Response of PE (1µM) in calcium free solution was observed in the absence and presence of Mn. Aq and control drug (verapamil) by using the technique followed previously by Qayyum *et al.* (2016). Any decrease in maximal response of PE was indicator for inhibition of intracellular calcium release.

STATISTICAL ANALYSIS

Data were stated as mean ± SEM. statistical significant (with P value <0.05) among various treatment groups was estimated using one-way ANOVA followed by Dunnett's test with confidence intervals 95% (CI). GraphPad prism (V5) was used to construct the graphs and statistical analysis.

RESULTS

Aqueous fraction of Morus nigra produced antihypertensive effect in fructose fed rats

A dose dependent fall in MAP and HR was observed with intravenous administration of Mn. Aq from 0.01 to 30 mg/kg in hypertensive rats. Mn. Aq significantly ($p<0.05$) reduced MAP (45.32±6.57% of control) and HR (52.10±3.80%). This significant effect was observed at 0.1 and 1mg/kg doses, respectively. Similarly dose dependent fall in MAP and HR was shown by verapamil with significant effect from 0.3mg/kg to higher doses (fig. 1).

Aqueous fraction of M. nigra relaxed rat's isolated thoracic aorta

Mn. Aq concentration dependently relaxed PE and high K^+ mediated contraction in rat's isolated aorta (EC_{50} value 3.72 and 1.25mg/ml, respectively) with maximum effect produced by 10 and 5mg/ml, respectively (fig. 2A, B).

Mn. Aq exhibited partial endothelium-dependent vaso-relaxant effect

Mn.Aq concentration-dependently caused relaxation in PE (1 µM) induced contraction in endothelial intact aorta with EC_{50} value 1.96mg/ml. A significant decrease in vaso-relaxant effect of Mn. Aq was seen in endothelial

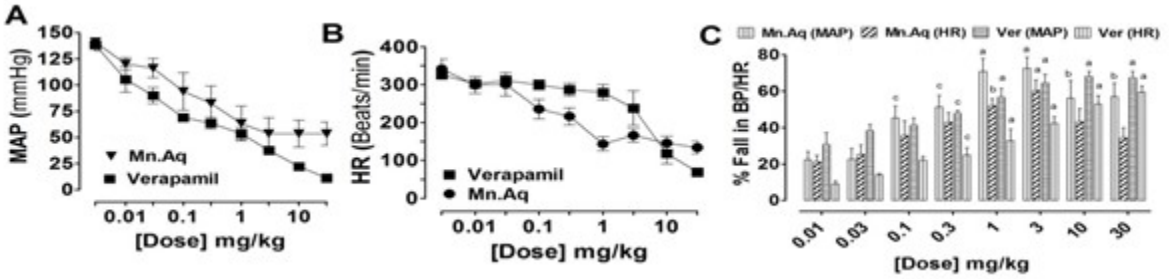

Fig. 1: Effect of aqueous fraction of *M. nigra* (Mn.Aq) (A) and verapamil (Ver; 0.01-30mg/kg) (B) on MAP and HR in fructose induced hypertensive rats. Bar chart is showing % age fall in MAP and HR with different doses of Mn. Aq and verapamil (C). Data presented as mean ± SEM. Symbols [a];P<0.001, [b];P<0.01 and [c];P<0.05 showing significance levels of MAP and HR.

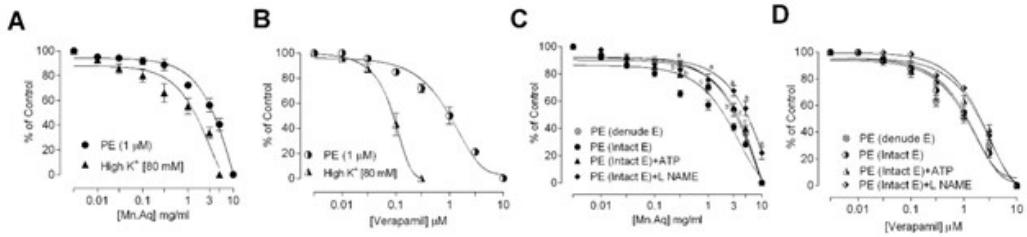

Fig. 2: Effects of Mn.Aq (A) and verapamil (B) on high K^+ and PE-induced contractions. Effects on denude and intact aorta in absence and presence of atropine (ATP; 1 µM) and Nitro-L-arginine methyl ester hydrochloride (L-NAME; 0.1 mM) (C, D). Where [a]; P<0.001, [b];P<0.01 and [c];P<0.05 is showing significant difference in response of Mn.Aq in denude aorta, atropine and L-NAME pre-treated aorta vs endothelial intact aorta.

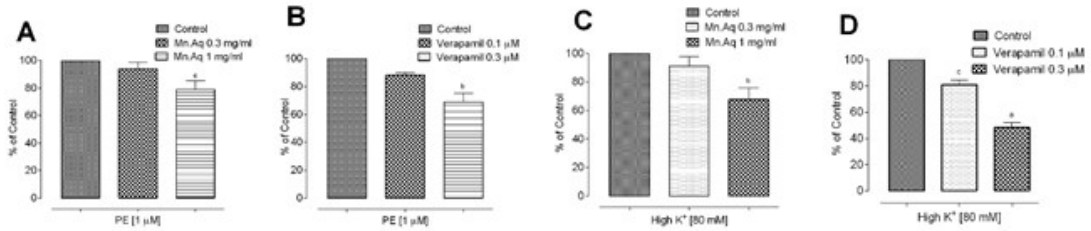

Fig. 3: Tissue response to PE (A, B) and high K^+ [80 mM] (C, D) in the absence and presence of Mn. Aq (0.3-0.1 mg/ml) and verapamil (0.1-0.3µM). Where [a];P<0.001, [b];P<0.01 and [c];P<0.05 vs control.

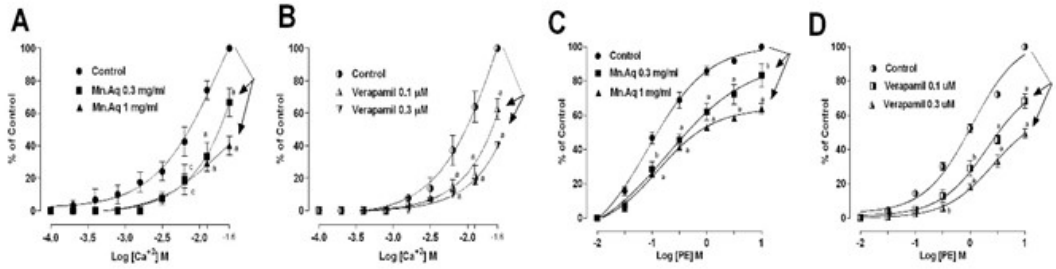

Fig. 4: Tissue response to Ca^{+2}(A, B) and phenylephrine (PE; log -2-log 1M) (C, D) in the absence and presence of Mn. Aq (0.3-0.1mg/ml) and verapamil (0.1-0.3µM) in Ca^{+2} free environment. Data are shown as means ± SEM of five experiments.

denude aorta and atropine (1µM) pre-treated endothelial intact aorta with EC_{50} value 3.41 and 3.12mg/ml, respectively. Whereas, vaso-relaxant effect of Mn. Aq was partially blocked with L-NAME (EC_{50} value 5.32mg/ml) (fig. 2C, D).

Mn.Aq inhibited receptor operated and voltage gated calcium channels

Pretreatment with Mn. Aq concentration dependently (0.3-1.0mg/ml) decreased tissue contractile response to PE. Significant ($p<0.05$) decrease with maximum effect (78.94±6.31%) was seen at 1mg/ml final bath concentration of Mn. Aq (fig. 3A). The response was almost similar to verapamil (fig. 3B).

Pretreatment with Mn. Aq concentration dependently (0.3 -1.0mg/ml) suppressed tissue response to high K^+.A significant ($p<0.01$) decrease in maximum response 66.64 ±8.2% was seen at 1mg/ml final bath concentration of Mn.Aq (fig. 3C), similar to verapamil (0.1-0.3µM) (fig. 3D).Furthermore, pretreatment with Mn. Aq also concentration dependently (0.01-10mg/ml) suppressed the tissue response to Ca^{+2}. A significant ($p<0.001$) decrease in maximal response 66.66±13.33% and 40.0±11.54% was seen at 0.3 and 1 mg/ml final bath concentration of Mn. Aq, respectively (fig. 4A), similar to verapamil (0.3 - 1 µM) (fig. 4B).

M.Aq inhibited release of calcium from intracellular calcium stores

Pretreatment with Mn. Aq concentration dependently suppressed the tissue response to PE in without Ca^{+2} medium. Significant ($p<0.01$) decrease in maximal response i.e., 83.4±6.77% and 63.84±3.17% was seen respectively at 0.3 and 1mg/ml final bath concentrations, (fig. 4C).

DISCUSSION

Present study was conducted in continuation to previous study. In which crude extract of *M. nigra* and derived fractions were investigated for presence of antihypertensive effect. Aqueous fraction of *M. nigra* (Mn.Aq) showed more distinct antihypertensive effects in normotensive rats and generates the perception that Mn.Aq is contributing majorly to hypotensive effect because to deposition of active constituent (s) in it. Therefore, Mn. Aq was further investigated to evaluate antihypertensive effects in fructose induced hypertensive rats. It has been established previously that continuous use of fructose rises blood pressure due to sympathetic stimulation, vaso-constriction and salt overload, etc. (Klein and Kiat, 2015). Intravenous administration of Mn. Aq in fructose fed hypertensive rats showed dependent fall in MAP and HR. That effect was similar to verapamil (Bühler *et al.*, 1982), indicating, the antihypertensive activity in Mn. Aq (Hall, 2015).

Antihypertensive drugs reduce the blood pressure with different mechanism of actions that eventually result either decrease in peripheral resistance or/and cardiac output (Adeneye *et al.*, 2006). A calcium channel blocker (CCB) decreases blood pressure by interfering with both of these blood pressure regulatory parameters (Michael and Hoffman, 2011). Therefore, decrease in blood pressure with the similar pattern to verapamil in invasive method, indicates not only the effectiveness of Mn. Aq in regulation of blood pressure but also create a perception about the probable presence of CCB like activity in Mn. Aq. To clarify the perception about the antihypertensive effect is either due to vascular effects, study was further proceeded on isolated tissue preparations because isolated tissue preparations allow studying the intrinsic tissue responses without any neuro-hormonal interruption (Yasin *et al.*, 2014). PE and high K^+ trigger constriction of vascular smooth muscles by increasing cytosolic Ca^{+2} through activation of voltage gated Ca^{2+} channels (Khan and Gilani, 2008) and by releasing intracellular Ca^{+2} from SR (Aslam *et al.*, 2016), respectively. The perception became stronger when Mn. Aq significantly suppressed high K^+ and PE induced contractions, CRCs with significant ($P<0.001$) suppression of maximal effect, analogous to verapamil (Malik *et al.*, 2017).

Complete relaxations of PE induced aortic contraction on same concentration of Mn. Aq (10mg/ml) in intact and denude aortic rings but with significant difference between the effects on both preparations, indicate minor involvement of endothelial-dependent relaxation through muscarinic receptors. The perception was strengthened when no prominent change in the vaso-relaxant effect was seen in the presence of atropine. However, partial blockade of vaso-relaxant effect of Mn. Aq in presence of L-NAME indicates the role of NO medicated endothelial dependent relaxation in the vaso-relaxant effect of aqueous fraction of *M. nigra* (Ghayur *et al.*, 2005).

CONCLUSION

Findings of current study designate the existence of antihypertensive activity in aqueous fraction of *M. nigra* with concentration-dependent vaso-relaxant effect predominantly mediated through endothelial-independent calcium channel blocking pathways accompanied by partial involvement of endothelium-dependent NOS mediated relaxation. Since the study validates the folkloric claims of *M. nigra* in hypertension, however identification of phytochemical constituents and their co-relation with antihypertensive potential are recommended in future prospective to get the better picture of *M. nigra.*

REFERENCES

Adeneye A, Ajagbonna O, Mojiminiyi F, Odigie I, Ojobor P, Etarrh R and Adeneye A (2006). The hypotensive

mechanisms for the aqueous stem bark extract of *Musanga cecropioides* in Sprague-Dawley rats. *J. Ethnopharmacol.,* **106**(2): 203-207.

Ahmad T, Khan T, Alamgeer and Shah AJ (2020). Juglone as antihypertensive agent acts through multiple vascular mechanisms. *Clin. Exp. Hypertens.,* **42**(4): 335-344.

Alamgeer, Chabert P, Akhtar M, Jabeen Q, Delecolle J, Heintz D, Garo E, Hamburger M, Auger C, Lugnier C and Kim HJ (2016). Endothelium-independent vasorelaxant effect of a *Berberis orthobotrys* root extract via inhibition of phosphodiesterases in the porcine coronary artery. *Phytomed.,* **23**(8): 793-799.

Aslam M and Sial AA (2014). Effect of hydroalcoholic extract of *Cydonia oblonga* Miller (Quince) on sexual behaviour of Wistar rats. *Adv. Pharmacol. Sci.,* **2014**: 282698.

Aslam N, Janbaz KH and Jabeen Q (2016). Hypotensive and diuretic activities of aqueous-ethanol extract of *Asphodelus tenuifolius*. *Bangladesh J. Pharmacol.,* **11**(4): 830-837.

Buhler FR, Hulthen UL, Kiowski W, Muller FB and Bolli P (1982). The place of the calcium antagonist verapamil in antihypertensive therapy. *J. Cardiovasc Pharmacol.,* **4**(Supp 3): S350-357.

Calvo M and Cavero R (2014). Medicinal plants used for cardiovascular diseases in Navarra and their validation from official sources. *J. Ethnopharmacol.,* **157**: 268-273.

Chaudhry MA, Mushtaq MN, Bukhari IA and Assiri AM (2021). *Ipomoea hederacea* Jacq.: A plant with promising antihypertensive and cardio-protective effects. *J. Ethnopharmacol.,* **268**: 113584.

Clark JD, Gebhart GF, Gonder JC, Keeling ME and Kohn DF (1997). The 1996 guide for the care and use of laboratory animals. *ILAR Journal,* **38**(1): 41-48.

Ghayur MN, Gilani AH, Afridi MB and Houghton PJ (2005). Cardiovascular effects of ginger aqueous extract and its phenolic constituents are mediated through multiple pathways. *Vasc. Pharmacol.,* **43**(4): 234-241.

Hall JE (2015). Guyton & Hall Physiology Review E-Book: Elsevier Health Sciences.

Hu GY, Peng C, Xie XF, Xiong L, Zhang SY and Cao XY (2018). Patchouli alcohol isolated from *Pogostemon cablin* mediates endothelium-independent vasorelaxation by blockade of Ca^{2+} channels in rat isolated thoracic aorta. *J. Ethnopharmacol.,* **220**: 188-196.

Khan A-U and Gilani AH (2008). Pharmacodynamic evaluation of *Terminalia bellerica* for its antihypertensive effect. *J. Food Drug Anal.,* **16**(3): 6-14.

Klein AV and Kiat H (2015). The mechanisms underlying fructose-induced hypertension: A review. *J. Hypertens.,* **33**(5): 912-920.

Kumar VR and Chauhan S (2008). Mulberry: Life enhancer. *J. Med. Plants Res.,* **2**(10): 271-278.

Malik A, Mehmood MH, Channa H, Akhtar MS and Gilani AH (2017). Pharmacological basis for the medicinal use of polyherbal formulation and its ingredients in cardiovascular disorders using rodents. *BMC Complement Altern Med.,* **17**(1): 142.

Michael T and Hoffman B (2011). Treatment of myocardial ischemia and hypertension. *In*: Brunton L, Chabner B and Knollmann B editors. *Goodman and Gilman's The pharmacological basis of therapeutics*. China, McGraw-Hill Medical, pp.745-788.

Padilha MM, Vilela FC, Rocha CQ, Dias MJ, Soncini R, dos Santos MH, Alves-da-Silva G and Giusti-Paiva A (2010). Antiinflammatory properties of *Morus nigra* leaves. *Phytother. Res.,* **24**(10): 1496-1500.

Qayyum R, Qamar HM-u-D, Khan S, Salma U, Khan T and Shah AJ (2016). Mechanisms underlying the antihypertensive properties of *Urtica dioica*. *J. Transl. Med.,* **14**(1): 254.

Trial PHA (2002). Major outcomes in high-risk hypertensive patients. *JAMA,* **288**(23): 2981-2997.

WHO AA (2011). The world traditional medicines situation. *Issues and Challenges,* **3**: 1-14.

Yasin M, Hussain Janbaz K, Imran I, Gilani AH and Bashir S (2014). Pharmacological studies on the antispasmodic, bronchodilator and anti-platelet activities of *Abies webbiana*. *Phytother. Res.,* **28**(8): 1182-1187.

Younis W, Schini-Kerth V, da Silva DB, Junior AG, Bukhari IA and Assiri AM (2020). Role of the NO/cGMP pathway and renin-angiotensin system in the hypotensive and diuretic effects of aqueous soluble fraction from *Crataegus songarica* K. Koch. *J. Ethnopharmacol.,* **249**: 112400.

Younis W, Schini-Kerth V, Junior AG, Nocchi SR, Silva DB and Roberts RE (2021). Endothelium-independent vasorelaxant effect of *Asphodelus tenuifolius* Cav. via inhibition of myosin light chain kinase activity in the porcine coronary artery. *J. Ethnopharmacol.,* **269**: 113693.

Zhou ZY, Xu JQ, Zhao WR, Chen XL, Jin Y, Tang N and Tang JY (2017). Ferulic acid relaxed rat aortic, small mesenteric and coronary arteries by blocking voltage-gated calcium channel and calcium desensitization via dephosphorylation of ERK1/2 and MYPT1. *Eur. J. Pharmacol.,* **815**: 26-32.