

Cardiac Depressant and Vasodilatory Effect of Flaxseed - Basis for the Medicinal Use in Hypertension

(Tekanan Jantung dan Kesan Kevasokembangan Biji Rami - Asas untuk Penggunaan Ubatan dalam Tekanan Darah Tinggi)

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ABSTRACT

Linum usitatissimum (Flaxseed) is known to be traditionally used for managing hypertension. In this study, we aim to provide a mechanistic basis for the medicinal use of Flaxseed in hypertension. The high-performance liquid chromatography (HPLC) analysis that we carried out during our study showed the presence of polar compounds (quercetin, nicotinic acid, and nicotinamide) in Flaxseed's crude extract (Fs.Cr; aqueous methanolic). In anesthetized rats, Fs.Cr reduced arterial blood pressure (BP) dose-dependently (10-100 mg/kg). When tested for its mechanism of action ex vivo, Fs.Cr inhibited both the force and rate of spontaneous contractions in the dose range of 1-10 mg/mL in isolated guinea-pig atria, similar to how verapamil, a standard Ca⁺² channel blocker does it. Further, Fs.Cr showed vasodilator effect against the contractions induced by phenylephrine (PE, 1 μM) in rat aortic ring preparations (concentration range: 1-10 mg/mL), whereas no effect was observed against the contractions induced by low K⁺ (25 mM) as well as high K⁺ (80 mM). This selective inhibitory effect of Fs.Cr against PE was tested for endothelium-dependent nitric oxide (NO) and/or cholinergic component involvement. The vasodilator effect of Fs.Cr against PE was retested in the absence and presence of atropine in endothelium (E)-intact and E-denuded aorta, but no significant shift was observed in the inhibitory effect of Fs.Cr. Further, Fs.Cr shifted the PE-induced concentration-response curves (CRCs) to the right in a dose-dependent manner (1 and 3 mg/mL). This effect was similar to that of prazosin. All these findings indicate that Flaxseed may mediate its antihypertensive activity by the alpha-1 receptor antagonist and Ca⁺² channel blocking-like activity, which may account for its efficacy in hypertension.

Keywords: Alpha-1 Receptor antagonist; aorta; atria; Flaxseed; hypertension; vasodilator

ABSTRAK

Linum usitatissimum (Biji rami) diketahui secara tradisi digunakan untuk mengawal tekanan darah tinggi. Tujuan kajian ini adalah untuk memberikan dasar mekanistik penggunaan Biji rami dalam tekanan darah tinggi. Analisis kromatografi cecair berprestasi tinggi (HPLC) yang kami jalankan menunjukkan adanya sebatian polar (quersetin, asid nikotinik dan nikotinamida) dalam ekstrak kasar Biji rami (Fs.Cr; metanolik berair). Pada tikus yang dibius, Fs.Cr mengurangkan tekanan darah arteri (BP) bergantung pada dos (10-100 mg/kg). Semasa diuji untuk mekanisme tindakannya ex vivo, Fs.Cr menghalang kekuatan dan kadar kontraksi spontan dalam julat dos 1-10 mg/mL di atria tikus belanda yang terencil, serupa dengan bagaimana verapamil, Ca⁺² standard penyekat saluran melakukannya. Selanjutnya, Fs.Cr menunjukkan kesan kevasokembangan terhadap kontraksi yang disebabkan oleh fenilefrina (PE, 1 μM) pada persiapan cincin aorta tikus (julat kepekatan: 1-10 mg/mL), sedangkan tidak ada kesan yang diamati terhadap kontraksi yang disebabkan oleh K⁺ rendah (25 mM) serta tinggi K⁺ (80 mM). Kesan penghambatan selektif Fs.Cr terhadap PE diuji untuk penglibatan komponen nitrat oksida (NO) dan/atau kolinergik yang bergantung pada endotelium. Kesan kevasokembangan Fs.Cr terhadap PE diuji semula dengan ketiadaan dan kehadiran atropin dalam aorta endotelium (E)-intact dan E-denuded, tetapi tidak ada perubahan yang ketara dalam kesan penghambatan Fs.Cr. Selanjutnya, Fs.Cr mengalihkan lengkung tindak balas kepekatan yang disebabkan oleh PE (CRC) ke kanan

dengan cara yang bergantung pada dos (1 dan 3 mg/mL). Kesan ini serupa dengan prazosin. Semua penemuan ini menunjukkan bahawa Biji rami dapat memediasi aktiviti antihipertensi oleh antagonis reseptor alfa-1 dan aktiviti seperti penyekat saluran Ca^{+2} , yang mungkin menyumbang kepada keberkesanannya dalam hipertensi.

Kata kunci: Antagonis reseptor alfa-1; aorta; atria; Biji rami; kevasokembangan; tekanan darah tinggi

INTRODUCTION

According to the World Health Organization (WHO 2014), 60% of all deaths in the world are from chronic diseases, and globally, one out of every three adults is a victim of high BP i.e. hypertension. Due to the widespread prevalence of this disease and its ability to damage vital organs, this disease poses a significant healthcare challenge (Cutler et al. 2008). The available treatment options require life-long use of multiple expensive drugs that are not only beyond the reach of the common people of this country but are also associated with multiple side-effects (Ghayur & Janssen 2010). This leads to non-compliance issues which subsequently lead to a person being at high risk of experiencing cardiovascular events (Mazzaglia et al. 2009).

Natural products offer a solution to this problem as they can simultaneously cater to multiple triggers of a disease with a relatively better safety profile than pure chemicals do. The use of natural products is also in line with the recent recommendations in the hypertension management guidelines. These guidelines endorse that lifestyle modifications should be considered to be among the key factors for the management of hypertension, especially by/for those people who are categorized as stage 1 hypertensive without other risk factors (Houle et al. 2013).

Among the dietary therapeutic strategies, one of the options is the use of functional foods that have a proven role beyond being nutritious. Flaxseed is one such food item that has been traditionally used in the treatment of cardiovascular diseases (Prasad 2009), and it has also shown success as an antihypertensive in clinical trials (Rodriguez-Leyva et al. 2013). Some studies have also shown Flaxseed to possess a very small hypotensive effect (Prasad 2009), whereas some other studies suggest Flaxseed to selectively be one of the most potent antihypertensives for hypertensive patients (Rodriguez-Leyva et al. 2013). Similarly, a meta-analysis identified the antihypertensive activity of Flaxseed when

consumed as a whole for more than 12 weeks (Khalesi et al. 2015). The inconsistent antihypertensive effects, seen during the clinical trial, suggest that background pharmacological screening, which helps in identifying both the therapeutic dose and the possible mechanism involved and which may save from significant drug interactions, was missing as far as the trial was concerned. The studies conducted, so far, on animals have provided limited information on Flaxseed's pharmacological mechanisms (Gogus & Smith 2010). For instance, a study showed that Flaxseed meal reduced systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR), but the reason for its antihypertensive effect was not provided, though they showed that Flaxseed maintained the intactness of the endothelial lining when compared to how the lining becomes denuded in the diseased control (hypercholesterolemia model) (Talom et al. 1999). Some other studies on the mechanism involved in Flaxseed's antihypertensive effects reported improvements in vascular contractile function (Dupasquier et al. 2006), increase in vasodilator levels (Sekine et al. 2007), inhibition of angiotensin I (Park & Velasquez 2012; Prasad 2013), and guanylate cyclase inhibition (Prasad 2004). The isolated tissue assembly set-up can give us information about the basic functional mechanism of Flaxseed and help us reflect on the possible involvement of the receptors and dose-response curve (Siddiqi et al. 2014). Therefore, in our study, we aimed to determine the pharmacological mechanism(s) of Flaxseed by using crude aqueous methanolic extract (Fs.Cr) on rat aorta (vascular function), guinea-pig atria (cardiac suppressant effect), and anesthetized rat model, to elucidate mechanistic basis for its medicinal use in hypertension.

MATERIALS AND METHODS

PLANT MATERIAL AND EXTRACTION

For the extraction procedure, the *Linum usitatissimum* L. (Flaxseed) plant material was used, which belongs to the

family *Linaceae* (Picture 1). An authentic herbal supplier, located in the local herb market of Karachi, Pakistan, was approached for purchasing the seeds. The Flaxseed that was purchased matched the description of its physical characteristics provided in the online plant list at [http://](http://www.theplantlist.org)

www.theplantlist.org. The seed was brown and oval, and it had a pointed tip, flat appearance, and a length of about 4 mm. The Flaxseed sample was given a voucher number (LU-SE-0819-001) and deposited in the Barrett Hodgson University's (BHU) herbarium for record.



PICTURE 1. Flaxseed (*Linum usitatissimum*)

For the preparation of the extract, first, the seeds were purified by removing all the dirt and other adulterants. Thereafter, it was finely powdered into powder by using an electrical chopper. To prepare the Fs.Cr from Flaxseed, approximately 1 kg of ground Flaxseed was cold macerated by using 30:70 v/v aqueous-methanol as a solvent for extraction (Taqvi et al. 2006). The extract was then dried at 40 °C in rotavapor to obtain Fs.Cr with a yield of 8%.

EXPERIMENTAL ANIMALS

Adult Sprague-Dawley rats (180-200 g) and guinea-pigs (450-500 g) were used for the *in vivo* and *ex vivo* studies, respectively. They were of a local breed and both sexes were used for the experiments. The animals were kept at a controlled temperature (23-25 °C) at an animal house in standard light and dark cycle. They were fed a standard

diet and the water supply to them was *ad libitum*. The protocols devised by the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington D.C., were adhered to for designing and conducting the study (Council 2010). The study protocol (BHU-ERC/Pharmacy-001/2020/PI - Dr Amber Hanif Palla) was also approved by the Ethical Research Committee (ERC) of BHU. The animal studies were designed by ensuring the care of animals in line with not only the national and international guidelines but also the institutional guidelines. The doses for the experiments were selected in accordance with the previously reported protocols (Shin et al. 2010).

CHEMICALS

The chemicals used for the experiments conducted during this study are the following: acetylcholine chloride

(Ach), isoprenaline hydrochloride, norepinephrine (NE), phenylephrine hydrochloride (PE), and prazosin. These chemicals were bought from Sigma Chemical Company, St. Louis, MO, USA), whereas pentothal sodium (thiopental) was obtained from Abbot Laboratories, Karachi, Pakistan.

PHYTOCHEMICAL ANALYSIS

The HPLC analysis of Fs.Cr was performed using the Shimadzu (Kyoto, Japan) Prominence 20-AT HPLC System, which was equipped with a binary pump (LC20AT), PDA detector (SPD-M20A), and Kromasil-100 C18 column (Teknokroma, 8 μ m, 25X0.46 mm) with a membrane filter (0.45 μ m) and syringe filter (0.45 μ m). The active constituents contained in Fs.Cr were not known erstwhile; however, as the initial screening showed peaks at earlier retention times (a characteristic of non-polar compounds; Ignat et al. 2011), some of the non-polar compounds whose peaks matched with the extract were used as standards.

For the HPLC analysis, Fs.Cr (2 mg) was dissolved in 2 mL mobile phase (methanol: acetonitrile: water: acetic acid; 40:20:39:1 v/v) and sonicated for 30 min to ensure homogenization. It was then filtered through the Millipore filter (0.45 μ m) to ensure uniform particle size. The flow rate was set at 0.8 mL/min, and the temperature of 25 °C was sustained in the column. The wavelength of the ultraviolet detector was set at 254 nm. As no reported standards were available for Fs.Cr at the time of the experiment, activity-guided standard drugs were run parallel to get an idea about the type of compounds present such as quercetin, luteolin, metronidazole, nicotinic acid nicotinamide, and ofloxacin.

MEASUREMENT OF BP IN ANESTHETIZED RATS

BP was assessed in an anesthetized rat according to the previously defined protocols (Hassan Gilani et al. 2005). At first, the rats were anesthetized by intraperitoneally administering them a dose of 70-90 mg/kg of thiopental sodium (Pentothal®). To provide assisted ventilation, tracheal cannulation was done by using a polyethylene tubing Pe-20. To administer drugs intravenously, cannulation of the right jugular vein was performed with the help of polyethylene tubing Pe-50. For recording the BP, a similar cannulation procedure by the polyethylene tubing was used in the left carotid artery but with the difference that it was pre-filled with heparinized

saline (60 IU/mL). This was done to prevent clogging as this tubing was connected to a pressure transducer (MLT 0380/D Reusable BP-Transducer), which was coupled to ML 224 Quad Bridge Amplifier and Power-Lab ML 4/25 data acquisition system (AD Instruments, Sydney, Australia). To prevent drying of the cannulated exposed surface, a piece of gauze which was moistened in warm saline was kept on it. Before administering any drugs/test material administration *via* IV in the jugular vein, heparinized saline (0.1 mL) was administered in the same tubing to lyse any blood clot that may have formed there and facilitate the smooth administration of the drugs and/or test material. At least 20 min were given for the heart rate to be stabilized after all the equipment and cannulation settings were put in place. After this, the test substance was injected intravenously, and the arterial BP was recorded. Before administering the next dose, BP was allowed to be restored to the resting level. The test material was considered antihypertensive if the steady-state values were reduced after the injection. The mean arterial pressure (MAP) was calculated as:

$$\text{MAP} = \text{Diastolic BP} + 1/3 \text{ pulse pressure}$$

$$\text{Pulse pressure} = \text{SBP} - \text{diastolic BP}$$

The test material was considered to alter MAP if a difference was evident between the resting value before and the lowermost reading after the administration of the test material.

EFFECT ON ISOLATED GUINEA-PIG ATRIA

To understand the mechanism of test material against spasmogens, a guinea-pig's right atrium was isolated and tested in isolated tissue assembly according to previously defined protocols (Hassan Gilani et al. 2005). The tissue baths of 20 mL volume were filled with Krebs's solution which was supplied with carbogen gas, and the temperature was maintained at 32 °C to mimic the mammalian body environment. A guinea-pig's right atrium has the natural automaticity for spontaneous beating due to a pacemaker (Taqvi et al. 2006). The atrium was stabilized for 45 min before the administration of any drug by using the weights of 1 g to maintain the resting tension. To record the effect of the drug on isometric contractions, the tissue was connected to an isometric sensor coupled with an amplifier. These were then

connected to the Power Lab and a computer. The effect of the test material was considered positive if the atrial rate and force of contraction were reduced when compared to their baseline values. The effect was reported as the percentage change in the baseline values.

EFFECT ON RAT AORTA PREPARATIONS

To study the effect of the test material on aortic preparations, previously defined protocols were used (Tep-Areenan & Sawasdee 2011). For this purpose, the rats were sacrificed by an overdose of inhalation of the anaesthetic Isoflurane. Once the rats' aorta was dissected, the fat and adipose tissues were excised, and then, 5 mm long rings were cut, which were then mounted individually in a tissue bath (5 mL) pre-filled with Krebs' solution. The tissue was connected *via* a thread to an isometric force transducer (Fort-10, WPI, UK), which was coupled to a bridge amplifier (Transbridge TBM 4M, WPI) and the Power Lab ML 845 data acquisition system. The transducer was used to measure the changes in tension. This was followed by stabilization of the aortic tissue for which Phenylephrine (PE was repeatedly administered to stimulate the alpha receptors that are present on the vessels). Isometric force transducers (Fort-10, WPI, UK) were used to record the contractions of the aorta, and they were further connected with a bridge amplifier (Transbridge TBM 4M, WPI) and the Power Lab ML 845 data acquisition system.

Once the aortic rings were stabilized, the effect of the drug was tested against different spasmogens including high K^+ (80 mM), low K^+ (30 mM), and PE-induced contractions. If the test material exhibited the inhibitory effect, it would indicate a calcium channel blocking (CCB) activity [L-type voltage-operated] and/or opening of K^+ channels, respectively. The inhibitory effect of the test material against PE-induced contractions would indicate Ca^{++} influx blocking activity mediated *via* receptor-operated calcium channels (Godfraind 1986).

STATISTICAL ANALYSIS

All data were analyzed along with its graphing and calculations by using the GraphPad Prism software version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). The data were presented as Mean \pm Standard error of the mean (SEM), whereas the geometric mean

(with 95% CI) was used to calculate the median EC₅₀ values. Nonlinear regression analysis was used for CRCs. p-values of less than 0.05 were considered statistically significant.

RESULTS

HPLC OF FLAXSEED EXTRACT

As is evident in Figure 1(a), HPLC showed the retention time (Rt) of Fs.Cr at 3.075 min, which was close to that of the compound nicotinic acid (2.955 min; 3.54 μ g/2 mg) (Figure 1(c)). This was followed by the Rt of Fs.Cr at 3.387 min (6.42 μ g/mg) which corresponded to that of the compound nicotinamide (3.327 min; Figure 1(d)). Finally, another peak was obtained at 5.608 min (9.53 μ g/mg) which was comparable to that of the compound quercetin (5.774 min; Figure 1(e)). There were two other peaks identified at 2.789 min (19.495 μ g/mg) and 3.633 min (6.195 μ g/mg) whose Rts matched with the synthetic drugs ofloxacin (an antibiotic) with Rt being 2.835 min (Figure 1(b)) and metronidazole (an antiameobic) with Rt of 3.699 min (Figure 1(f)).

ANTIHYPERTENSIVE EFFECT OF Fs.CR

The effect of Fs.Cr on invasive BP was assessed in anesthetized rats (n = 3). Fs.Cr decreased MAP by $20 \pm 2\%$, $32.5 \pm 2.5\%$, and $47 \pm 2\%$ mm Hg for the doses of 10 mg/kg, 30 mg/kg, and 100 mg/kg, respectively. Figure 2(A) shows the tracing of the effect of Fs.Cr on BP in an experiment, whereas the combined hypotensive effect of Fs.Cr on MAP in our experiment is depicted in Figure 2(B).

EFFECT OF Fs.CR ON RATE AND FORCE OF CONTRACTION IN GUINEA-PIG ATRIA

To assess the effect of Fs.Cr on the contractility of the heart, the isolated guinea-pig paired atria was used. As is evident in Figure 3(A), Fs.Cr inhibited the spontaneous rate and force of the contractions which were concentration-dependent with the EC₅₀ values being 4.2 mg/mL (95% CI: 3.1-5.2; n = 3) and 3.7 mg/mL (95% CI: 4.6-5.2; n = 3), respectively. Verapamil similarly inhibited the force and rate of contraction with respective the EC₅₀ values being 2.84 μ M (2.46-2.98; n = 3) and 3.14 μ M (2.86-3.42; n = 3) as shown in Figure 3(B).

VASODILATOR EFFECT OF Fs.CR ON RAT AORTA

The baseline evaluation of Fs.Cr up to the tested dose of 10 mg/mL showed that the extract did not exhibit any vasoconstrictor effect in the rat aortic rings (tested data not shown). When tested against the vasoconstrictors such as low & high K^+ (25 and 80 mM) and PE (1 μ M) which induce vasoconstriction, Fs.Cr relaxed the vessels against PE with the EC_{50} values of 5.7 mg/mL (4.2-7.4; $n = 3$), whereas no inhibitory activity was observed against the contractions induced by low and high K^+ (Figure 4(A)). The inhibitory effect against PE (1 μ M)-induced vascular contractions was similar to that of prazosin, a standard competitive α_1 receptor antagonist with EC_{50} values being 0.2 mg/mL (0.15-0.28; $n = 3$) as shown in Figure 4(B). To further confirm this, CRCs were plotted after the pre-incubation of the rat aortic rings with Fs.Cr (1 and 3 mg/mL). Expectedly, Fs.Cr at the doses of 1 and 3 mg/mL shifted the PE-CRCs to the right in a parallel manner without the suppression of the maximum response (Figure 5(A)). This rightward parallel shift in the PE-curves was similar to that obtained in the case of pre-treatment of prazosin (0.01-0.03 μ M; $n=3$) as shown in Figure 5(B), thus, confirming the presence of a competitive α_1 antagonist type.

No significant difference (> 0.05) was observed in the EC_{50} values when Fs.Cr was tested in the E-intact and E-denuded aorta (Figure 6). Atropine pre-incubation did not change the EC_{50} values (Figure 6), thus indicating a lack of the anticholinergic and cGMP-dependent mechanisms' involvement in blood vessels.

DISCUSSION

Flaxseed has traditionally been used to manage cardiovascular disorders including hypertension (Prasad 2019). Recently, Flaxseed has gained a lot of attention because of its numerous cardiovascular health benefits that are attributed to the soluble fibres, α -linolenic acid (ALA) and lignans (phytoestrogens) in it (Jhala & Hall 2010). Several clinical trials have been conducted to assess its antihypertensive effects (Rodriguez-Leyva et al. 2013), but the data available about the pharmacological rationale for its effectiveness is limited.

In view of this, we evaluated the antihypertensive effect of Fs.Cr in an anesthetized rat and studied the possible mechanisms in an isolated tissue assembly set up using guinea-pig atria and rat aorta.

Our study shows that Fs.Cr when administered intravenous (IV), reduces the rat's arterial BP dose-

dependently. This finding of ours is consistent not only with Flaxseed's known traditional use but also with the previous reports of research (Prasad 2009, 2004) that found Flaxseed to significantly reduce MAP in animals when administered IV (Prasad 2009), which is why it was possibly used in clinical trials.

Surprisingly, the results of the clinical trials were inconclusive. In some of these trials, there was a marked antihypertensive response (Rodriguez-Leyva et al. 2013), whereas such a response was either marginal or absent in others (Khalesi et al. 2015; Prasad 2009). The inconsistency of Flaxseed (difficulty in dissolution with water) has been reported earlier by Palla et al. (2016), which was also supported by the previous findings in this regard where Secoisolariciresinol diglucoside (SDG)-rich Flaxseed significantly lowered SBP in animals only in the presence of a high-fat diet administration (Park & Velasquez 2012), whereas in the case of IV administration, the effects were marked and independent of high fat reflecting an issue in its oral absorption (Park & Velasquez 2012). It is proposed, therefore, that our future studies will address this aspect of inconsistent effects because it is possible to derive more health-related benefits from this plant if we understand the optimal method of its intake.

To evaluate Flaxseed's mechanism of action, isolated tissue preparations are routinely used as then the tissues' response is not interfered with by the reflex actions of the body (Ajay et al. 2007). Rat thoracic aorta is routinely used for assessing the effect on underlying pharmacodynamics of BP-lowering effects, i.e., vascular resistance. Flaxseed extract was tested against low and high K^+ , as well as, against PE-induced contractions in rat aorta to distinguish whether Flaxseed mediates its antihypertensive activity by the K^+ channels' opening, or the blocking of voltage-operated channels and/or the receptor-operated calcium channels, respectively (Okmura et al. 1993). As Fs.Cr selectively inhibited the PE-induced contractions in the aortic rings with no significant inhibition against low and high K^+ , it indicated that it may be acting through the receptor-operated calcium channels. The effect of these receptor-operated channels was similar to the α_1 receptor owing to the former's rightward parallel shift (Doggrell 1992). The α_1 receptor's blocking of the activity of Fs.Cr was confirmed when the pre-incubation of the aortic rings with Fs.Cr caused the rightward but parallel shift in the PE-CRCs that was similar to the shift in the case of prazosin, a competitive α_1 antagonist (Doggrell 1992).

This is an important finding because functional data of this sort has not been reported so far. It reflects on the basic mechanism by which Flaxseed may contribute to its antihypertensive effect. It has been reported through a series of clinical trials that α 1-receptor antagonists lower total peripheral resistance and reduce left ventricular hypertrophy even as they improve glomerular filtration rate (Schmieder et al. 1996). Together, these contribute to the improvement in BP. Thus, the inhibitory effect of Flaxseed may be due to the competitive α -1 antagonist effect that may cause vasodilation, the subsequent decrease in peripheral vascular resistance, and ultimately contribute to a reduction of BP.

In our experiments, we did not observe any difference in the effects on intact versus denuded aorta. Possibly, this means that for the tested doses, Flaxseed does not mediate its hypotensive effect *via* the L-arginine-NO pathway. This is consistent with the previous reports where SDG, lignin isolated from Flaxseed, did not mediate its effect *via* the stimulation of the NO synthase pathway and rather mediated the hypotensive effect by the cGMP pathway (Prasad 2004). Similarly, dietary Flaxseed could also not attenuate endothelial vasodilation mechanism when administered along with a cholesterol diet for a prolonged time (Dupasquier et al. 2006). As can be noted from the results, the α -1 antagonist effect is mediated at a higher dose of 1 mg/mL. Therefore, the possibility of other mechanisms cannot be excluded.

As both the blood vessels and the heart simultaneously play a role in maintaining BP, (BP = cardiac output \times peripheral vascular resistance [PVR]), the effect of Fs.Cr was studied on isolated guinea-pig atria to understand whether Fs.Cr also affects cardiac contractility. It was found that Fs.Cr reduced the rate and force of contraction similar to how verapamil, a non-specific Ca^{++} channel blocker that predominantly acts on L-type Ca^{++} channels, does it. This CCB-like activity is consistent with our previous studies in which Fs.Cr mediated its antispasmodic activities by blocking the Ca^{++} channels (Palla et al. 2015). It is interesting to note that Fs.Cr did not exhibit any CCB-like activity in vessels as it did not inhibit high K^{+} -induced contraction. It is possible that Fs.Cr mediates tissue-specific actions as reported in the case of other plant materials (Xiao et al. 2006), hence, its CCB activity is specific for the heart and gut tissues but not so in the vessels. Understandably, the difference in terms of the presence of a different type of receptors could be attributed to this specificity as the heart predominantly

has L-type Ca^{++} channels that are highly responsive to verapamil, whereas the blood vessels predominantly have T-type Ca^{++} channels (Katz 1996). Thus, it seems that Fs.Cr may mediate its CCB-like activity *via* the L-type Ca^{++} channels that could be attributed to its cardio-depressant effect caused by a reduction in the rate and force of the contraction of the heart in guinea-pig atria and reduction of SBP in the anesthetized rat. This coupled with the α -1 antagonist effect on blood vessels may be responsible for the antihypertensive activity of Fs.Cr. The antihypertensive effect of Fs.Cr could be more marked in hypertensive rats because of increased expression of the L-type Ca^{++} channels in them and the consequent Ca^{++} influx leading to abnormal arterial tone (Sonkusare et al. 2006).

Besides these mentioned antihypertensive mechanisms, several other mechanisms have been reported that are attributed to the antihypertensive activity of Flaxseeds including the angiotensin-converting enzyme inhibition (ACEI) and guanylate cyclase inhibition-like activities (Prasad 2013, 2009).

Besides, the extract that we used in the experiments conducted for this study, various other forms of Flaxseed, including milled Flaxseed, Flaxseed oil, defatted Flaxseed meal (rich in protein), Flaxseed protein hydrolysate (FPH), and SDG (lignin of Flaxseed), have been studied previously for deciphering their antihypertensive mechanisms. SDG has been shown to mediate antihypertensive effect *via* the guanylate cyclase inhibition (Prasad 2013), whereas both Flaxseed oil and SDG have shown ACEI activity (Ogawa et al. 2009; Prasad 2013). Flaxseed oil has been shown to reduce BP with simultaneous production of PGI₂ and NO metabolites, possibly, through the bradykinin stimulation (Sekine et al. 2007). Milled flaxseed has shown an antihypertensive effect that corresponds to the level of oxylipins, and this activity was associated with the presence of alpha-linolenic acid (ALA) in Flaxseed, which is a component found in high quantities in Flaxseed oil and whole seed (Caligiuri et al. 2014). FPH has been shown to exhibit antihypertensive activity in SHR by ACEI and renin inhibition (Udenigwe et al. 2009). Hence, taken together as a whole, and based on the findings of this study, we are reporting for the first time that Flaxseed extract mediates its antihypertensive effect as CCB on the heart and vasodilation that is mediated *via* the α -1 antagonism activity in the blood vessels. This coupled with reasons surfaced in the previous reports for the vasodilatory activity mediated *via* the guanylate cyclase

inhibition, PGI₂, and NO production, and ACE and renin inhibition activity may be the contributory factors for the overall antihypertensive effect of Flaxseed (Kumeshini et al. 2016). Therefore, Flaxseed as a whole has immense antihypertensive potential provided we use an optimal method for its administration that ensures absorption of the polar as well as the non-polar constituents.

This study is the first to report the fingerprint analysis of Flaxseed extract using HPLC. So far, there are no standards reported for the use of Flaxseed extract, which was obtained by using 70:30 ethanol-water. The components so far reported for flaxseed are representative of its polar constituents such as ALA. Therefore, for the extract, we identified the compounds by matching the peaks with the closest retention times of known compounds which corresponded to nicotinic acid, nicotinamide, quercetin, ofloxacin, and metronidazole. The antihypertensive mechanism observed in this study is relatable to the HPLC's findings, as quercetin, an

antioxidant compound found in Fs.Cr, has previously shown antihypertensive activity in both rodents and hypertensive humans (Edwards et al. 2007) along with ACEI-like activity as the possible antihypertensive mechanism (Mackraj et al. 2008). Quercetin has also been reported to mediate antihypertensive activity *via* the increased endothelial nitric oxide synthase (eNOS) activity and consequent increase in NO (Sanchez et al. 2006; Yamamoto & Oue 2006). In our study, Fs.Cr did not mediate its vasodilatory activity *via* the NO pathway, possibly, because we used normotensive Sprague Dawley rats, whereas quercetin effects were observed only in SHR and not in Wistar-Kyoto (WK) rats. Besides quercetin, nicotinic acid, identified as one of the compounds in Fs.Cr, has been shown to reduce BP in hypertensive humans (Gadegbeku et al. 2003). Interestingly, a novel combination of quercetin and nicotinic acid has exhibited an antihypertensive effect in SHR but not in WK rats (Wang et al. 2014). This aspect indicates that

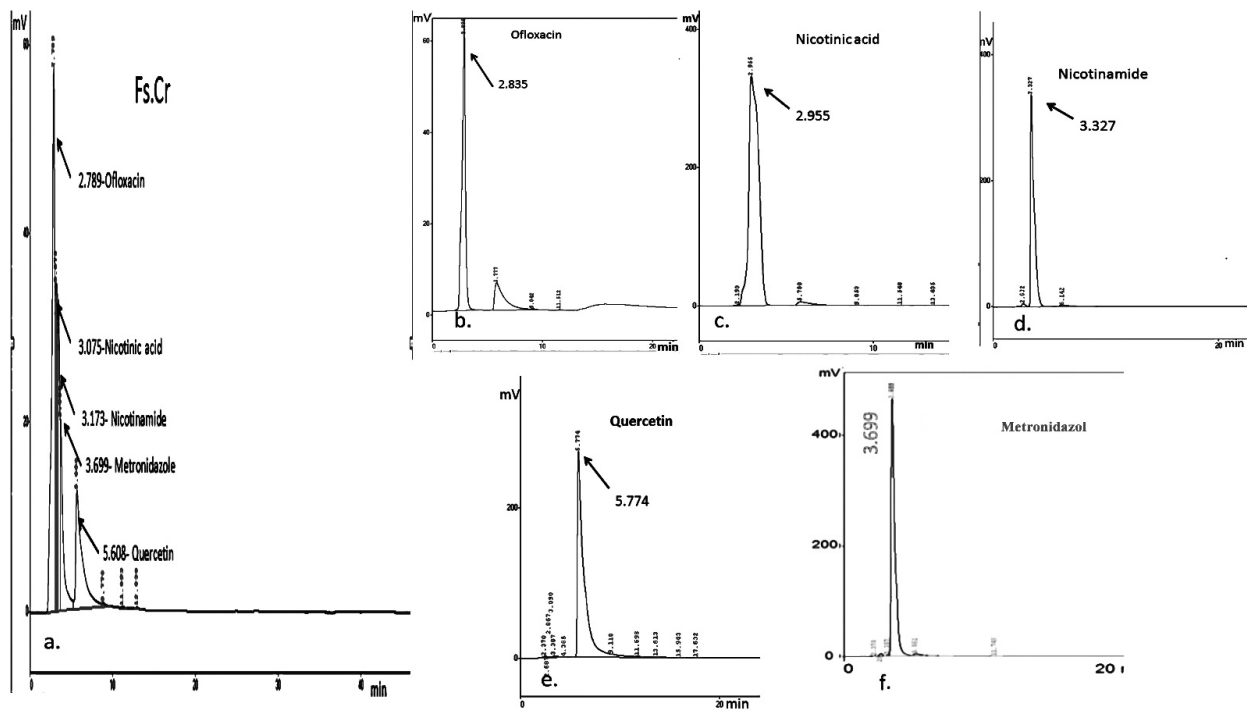


FIGURE 1. High performance liquid chromatography (HPLC) fingerprints of (a) Fs.Cr. Peaks of Fs.Cr resemble with nicotinic acid (Rt=2.955), nicotinamide (Rt=3.327) and quercetin (Rt=5.774). Other unidentified compounds matching synthetic compounds ofloxacin (Rt=2.835) and metronidazole (Rt=3.699). Peaks identified in Figure indicate retention time (in min) of respective compounds, (b) peaks of Fs.Cr resemble with ofloxacin (Rt=2.835), (c) nicotinic acid (Rt=2.955), (d) nicotinamide (Rt=3.327), (e) quercetin (Rt=5.774) and (f) metronidazole (Rt=3.699). Peaks identified in Figure indicate retention time (in min) of respective compounds

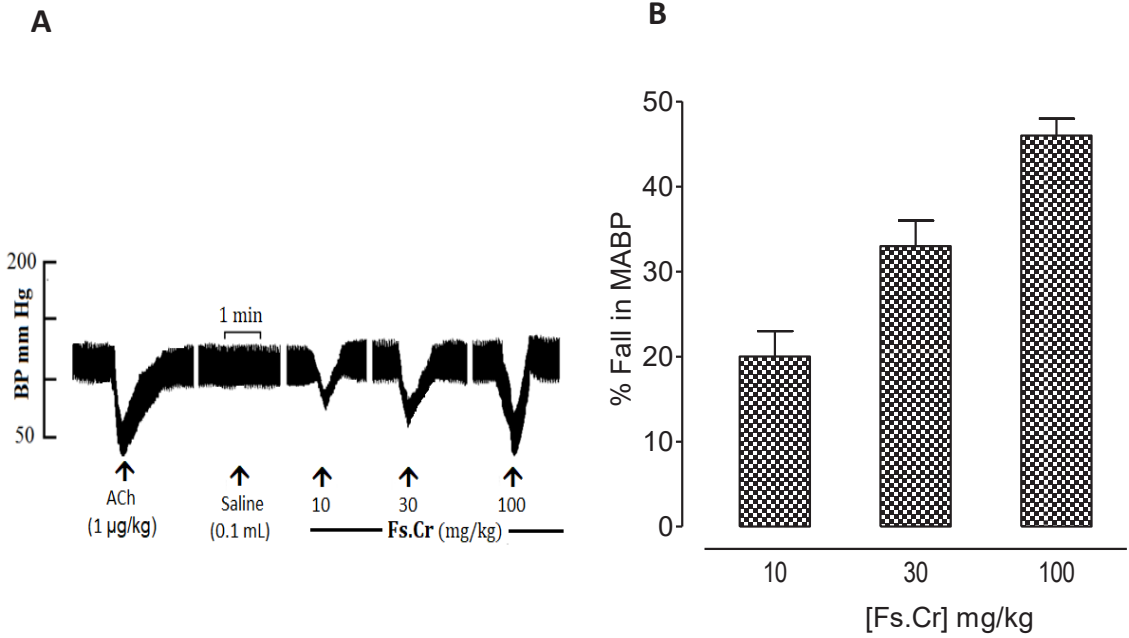


FIGURE 2. Panel (A) shows a typical tracing of Flaxseed crude extract (Fs.Cr) blood pressure (BP)-lowering effect and Panel (B) shows a bar chart representing hypotensive effect of Fs.Cr on mean arterial blood pressure (MAP) in anesthetized rats. The dose was administered after the response to the preceding one had returned to normal. Values shown represent mean \pm SEM, n=3

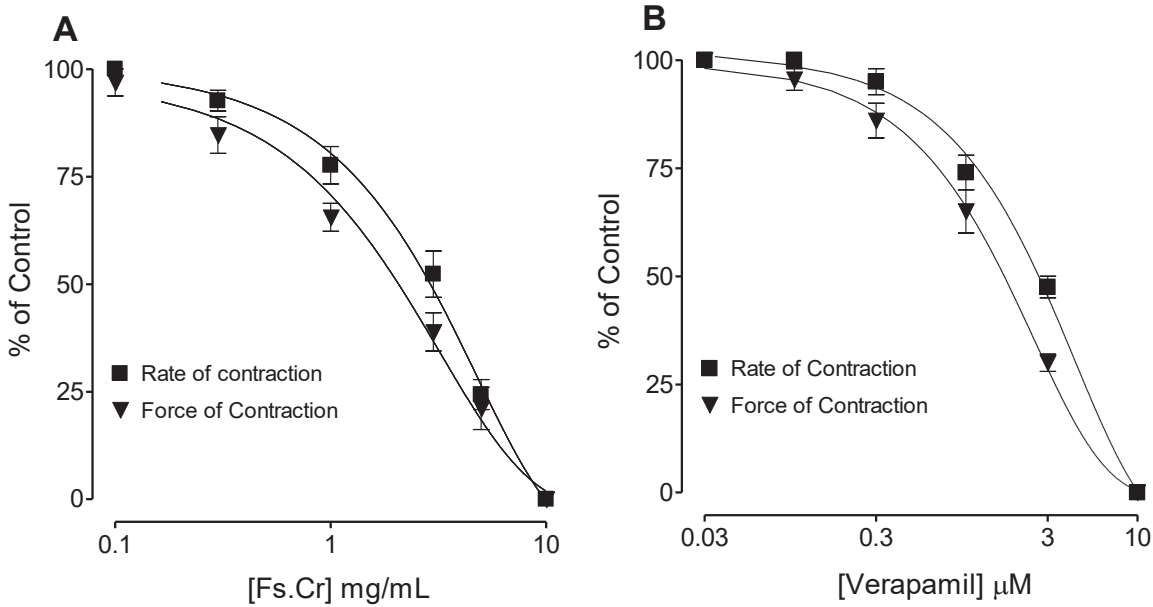


FIGURE 3. Inhibitory effect of Flaxseed crude extract (Fs.Cr) (A) and verapamil (B) on force and rate of spontaneous contractions in isolated guinea pig right atria. Values shown are mean \pm SEM, n = 3

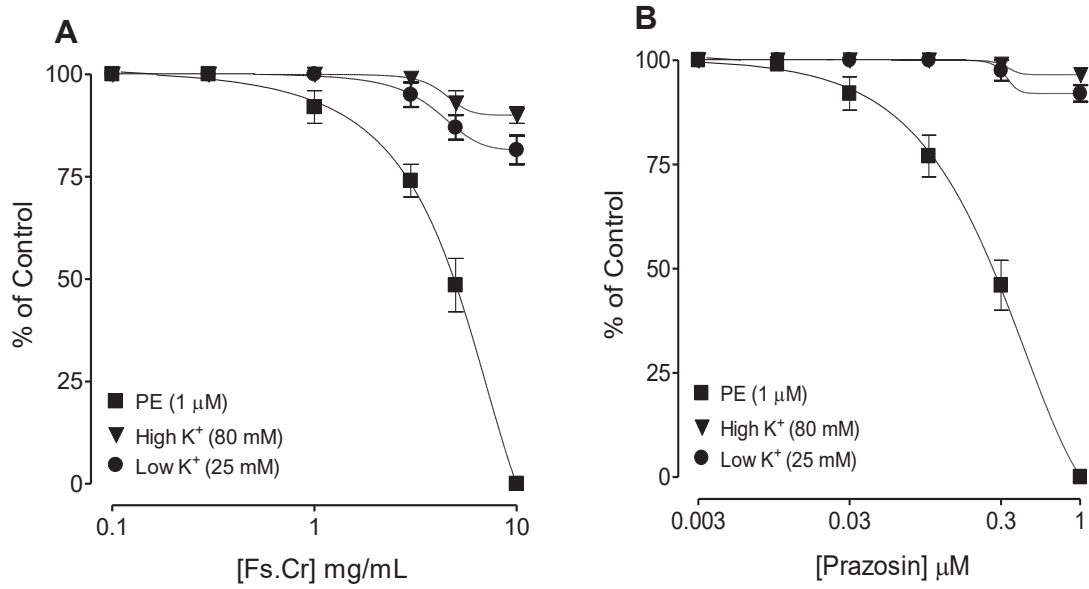


FIGURE 4. Concentration-dependent relaxant effects of (A) Flaxseed crude extract (Fs.Cr) and (B) prazosin on Low K⁺, high K⁺ and phenylephrine (PE)-induced contractions in isolated rat aortic ring preparations. Values shown are mean \pm SEM, n=3

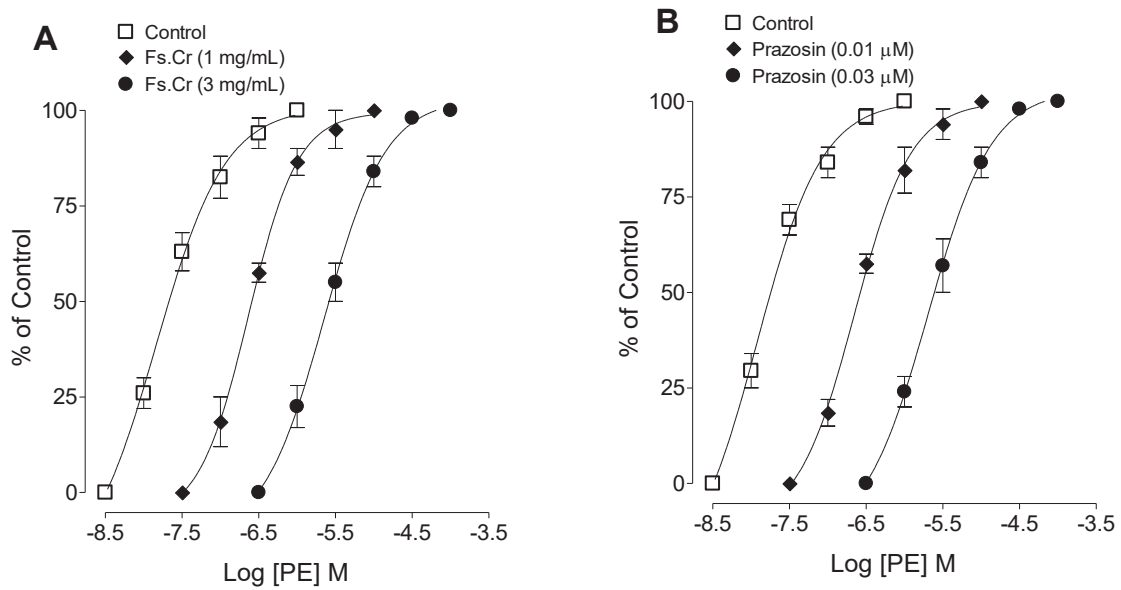


FIGURE 5. Concentration-response curves of phenylephrine (PE) in the absence and presence of different concentrations of (A) Flaxseed crude extract (Fs.Cr) and (B) prazosin in isolated rat aortic ring preparations. Values shown are mean \pm SEM, n=3

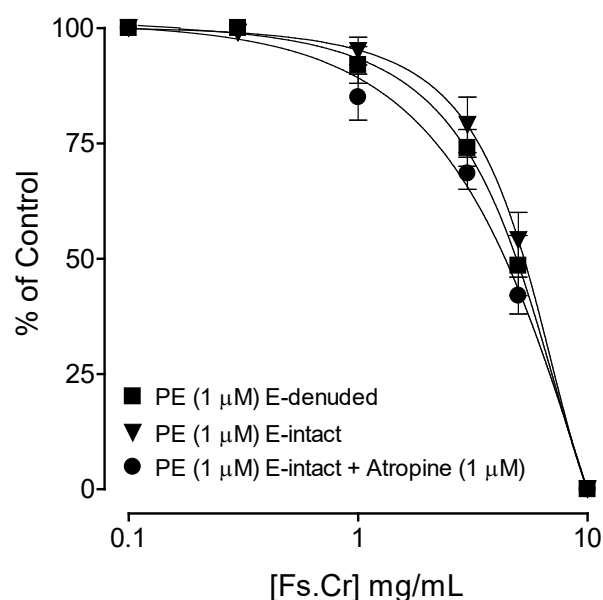


FIGURE 6. Concentration-dependent relaxant effects of Flaxseed crude extract (Fs.Cr) on phenylephrine (PE)-induced contractions in the absence and presence of atropine (1 μ M) in endothelium (E)-intact and E-denuded isolated rat aortic ring preparations. Values shown are mean \pm SEM, n=3

Flaxseed, by virtue of their active constituents, may have both the tissue- and specimen-specific effects as we have mentioned earlier that CCB-like activity was evident in atria and not in aorta. Therefore, our future studies may be focussed on assessing the mechanisms of Flaxseed in different species and on different tissues.

This study has certain limitations. We were not able to demonstrate the pharmacological mechanism attributed to inhibitory activity in the guinea-pig atria. In our future studies, we will aim to determine the possibility of beta-blocking and/or the presence of the anticholinergic component in the atria to confirm these mechanisms. Another limitation was that we selected rats of both sexes. The effect of female hormones has been evident in the prevention of hypertension. It has been reported earlier that female rats who were ovariectomized became hypertensive in four weeks (Kumeshini et al. 2016). However, hypertension in ovariectomized rats was evident only when fed with a 2% cholesterol diet, indicating

that the protection from hypertension in them was attributed to a reduction in their dyslipidaemic parameters (Kumeshini et al. 2016). Hence, the hormonal aspect in the female rats used may have had a lesser impact on the findings of this study, as this study is a mechanistic study, and we performed it on normotensive rats, which were on a normal diet. Nevertheless, earlier studies have consistently shown a difference in the cardiovascular disease parameters in males and females with the male and postmenopausal women being at greater risk of cardiovascular diseases when compared to premenopausal or adolescent females (Blenck et al. 2016). In accordance with the new guidelines issued by the National Institute of Health about considering the biological sex as a potential experimental variable in both animal and human studies focused on cardiovascular diseases (Blenck et al. 2016), our future mechanistic studies would certainly consider male and female animals as separate groups.

CONCLUSION

In conclusion, Fs.Cr exhibits BP-lowering, cardio-depressant, and vasodilator effects. The mechanism involved in vasodilation by Flaxseed is possibly mediated by competitive inhibition of the alpha-1 adrenergic receptors. However, further studies are needed to elucidate the cardiac depressant effects.

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