Mechanism of vasorelaxant activity of a fraction of root extract of
*Sesamum indicum* Linn.

P Suresh Kumar, J S Patel & M N Saraf*

Department of Pharmacology,
The Bombay College of Pharmacy, Sunder Nagar, Kalina, Santacruz (East), Mumabi 400 098, India

Received 10 April 2007; revised 15 April 2008

The petroleum ether soluble fraction (SIPE) of the root extract of *S. indicum* was evaluated for the vasorelaxant activity using isolated rat aorta. SIPE up to 180 µg/ml concentration significantly inhibited phenylephrine- and KCl-induced contraction to the extent of 98.13 ± 6.37 and 70.19 ± 3.43% respectively in isolated rat aorta in a concentration dependent manner. The vasorelaxant activity was not blocked by propranolol (10 µM), atropine (1 µM) indomethacin (10 µM) and glibenclamide (10 µM). Influence of SIPE on phenylephrine-induced contractions in aortic preparations in absence of functional endothelium and on pre-incubating the tissue with L-NAME (300 µM) or methylene blue (10 µM) was also studied. SIPE at 180 µg/ml concentration could elicit partial relaxation in presence of L-NAME or methylene blue to the extent of 34.26 ± 6.13 and 25.66 ± 10.95% respectively. However, in absence of functional endothelium, SIPE exhibited little relaxation to the extent of 6.70 ± 4.87%. These studies revealed that the vasorelaxant activity of SIPE was chiefly mediated through endothelium-dependent pathway.

Keywords: Aorta, Endothelium-dependent, *Sesamum indicum*, Vasorelaxant

Medicinal plants have been used for various ailments by alternate system of medicine and folklore treatments. However, many ethnopharmacological uses are yet to be justified scientifically for the rationale and safe use. Further, the phytoconstituents present in the plants are one of the sources for new chemical entities in drug discovery.

In search for medicinal plants for spasmolytic activity, the roots of *Sesamum indicum* Linn. (Marathi and Hindi: Til; Sanskrit: Pavitra; English: Sesame; Family: Pedaliaceae) has been selected on the basis of Ayurvedic and folklore use. The roots of *S. indicum* are known for their ethnomedical uses like enrichment of the blood, tonic to hair, useful in sore throat, spleen troubles, and to treat strangulation. *S. indicum* is one of the most important crops cultivated throughout the world. There are many pharmacological investigations on sesame seeds and sesame oil. However, little research has been reported on the roots, an under-utilized part of sesame.

The roots of *S. indicum* are reported to contain 1,4-naphthoquinone derivatives; anthralesamone A, anthrassamone B, anthralesamone C, 2-(4-methyl-pent-3-yl)anthraquinone and *(E)-2-(4-methyl-penta-1,3-dienyl)anthraquinone*<sup>10</sup>. The naphthoquinones from the roots of *S. indicum* are reported to exhibit *in vitro* antimicrobial activity<sup>8,11</sup>. The roots were used to treat bronchial asthma and the preliminary experiments revealed that root extract exhibited spasmolytic activity. Hence, an attempt has been made to evaluate the vasorelaxant activity of *S. indicum* in the present investigation.

Materials and Methods

*Plant material* — Fresh roots of *S. indicum* were collected from the fields at Thiruvallur, near Chennai, Kanchipuram district, during February 2006 after harvesting the mature seeds. The roots were then thoroughly washed with water to remove adhering soil and dried under shade. A voucher specimen was deposited at the Department of Pharmacology, Bombay College of Pharmacy, Kalina, Mumbai. The dried roots were coarsely powdered and stored in airtight, non-toxic polyethylene bags until used.

*Chemicals* — Hexane, methanol, ethylacetate were purchased from M/s Qualigens, Mumbai. L-NAME was purchased from Sigma Aldrich, USA. Methylene blue was purchased from SD Fine Chemicals,
Mumbai. Phenylephrine, glibenclamide, and propranolol were generous gift from Novartis India Ltd, Wockhardt laboratories and Cipla Ltd respectively. Acetylcholine chloride and atropine were purchased from Loba Chemie, Mumbai. Indomethacin was obtained from the commercially available capsules (Inocin®) after extraction of the contents of a capsule with chloroform. All salts used for physiological salt solution were procured from SD Fine Chemicals, Mumbai.

Animals — Sprague-Dawley rats (250-300 g) of either sex were obtained from the animal house of the Bombay College of Pharmacy and were housed under standard husbandry conditions, 26°±2° C, 60±20% RH, 12:12 hr dark/ light cycle, fed on commercial diet and water ad libitum. Animal studies were approved by Institutional Animal Ethics Committee and carried out in accordance with the Guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals.

Extraction and fractionation procedure — The coarse powder of roots of S. indicum (200 g) was extracted with 800 ml methanol by maceration in dark for 48 hr. It was then pressed, filtered and subsequently the extraction procedure was repeated with the marc (400 ml of methanol). The methanolic extracts (SIME) were combined and concentrated to sticky mass (~5% w/w) under reduced pressure. The sticky mass (3 g) was diluted with 50 ml of water and partitioned with petroleum ether (75 ml × 2), ethylacetate (75 ml × 2) to obtain petroleum ether soluble fraction (SIPE, 0.2% w/w) and ethylacetate soluble fraction (SIEA) respectively. These fractions and the remaining aqueous portion (SIW) were concentrated at room temperature until constant weight was obtained. Residual solvent was dried and the dried extract or the fractions of the extract were dissolved in DMSO and later diluted with water. The final concentration of DMSO in the organ bath volume was always < 0.1%.

SIME and SIPE were found to possess vasorelaxant activity. The vasorelaxant activity was found to be better with SIPE than SIME and other fractions. Hence, SIPE was selected for detailed investigations.

Phytochemical characterization — SIPE was subjected to general phytochemical analysis for presence of carbohydrates, proteins, aminoacids, tannins and phenolics, flavonoids, alkaloids, anthraquinones, cyanogenetic glycosides, saponin glycosides, and steroidal nucleus using the standard methods12,13. The UV-visible and IR spectrum of SIPE was also analyzed and compared with the reported literature. Further, aliquots of SIPE were applied on to TLC plates (silica gel G60 F254, E. Merck) using Linomat applicator 5 and the chromatogram was developed using ethyl acetate:hexane (2:8) as the solvent system. The separated bands were densitographically quantified at 435 nm a using CAMAG Scanner 3 with the help of WinCATS software. The orange-yellow band was isolated from the preparative TLC and IR fingerprint was recorded and compared with the reported data on anthraquinones from this plant.

Isolated rat aortic ring preparation and tension recording — Rats were killed by cervical dislocation and then exsanguinated by carotid artery transection. The thoracic aortas of the rats were rapidly and carefully dissected and placed into ice-cold Krebs-bicarbonate buffer (PSS, pH 7.4) [composition (mmol/L): NaCl, 113; KCl, 4.8; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25; glucose, 5.7]. Thoracic aorta was cleaned off adhering fat and connective tissue and cut into rings (~5 mm length). All dissecting procedures were done with extreme care to protect the endothelium from inadvertent damage. In order to study endothelium-independent vascular responses, some rings were rubbed intentionally on the internal surface with a thin cotton swab to remove the endothelium. These rings were placed in a jacketed organ bath chamber of 20 ml capacity of borosilicate glass, and mounted on two small stainless steel hooks, one of which was connected to the organ bath and the other to a force transducer (FT050/D, ADInstruments, Australia). The organ bath was filled with PSS maintained at 37°C and gassed with a mixture of 95% O₂ and 5% CO₂. The rings were allowed to equilibrate for 1 hr under a resting tension of 1 g using micromanipulator until a constant base force was established. During this time, the bathing medium was changed every 15 min. The isometric tension was measured using a force transducer and recorded digitally using a Data Acquisition System (Powerlab 8SP, ADInstruments, Australia) and then stored and analyzed with a computer program (Chart V5.2.1, ADInstruments, Australia).

After the equilibration period, all tissues were exposed repeatedly to 80 mM KCl solution until responses became stable, in order to test their
contractile capacity. The presence of functional endothelium was confirmed by the ability of acetylcholine (1 µM) to induce ≥ 50% relaxation of rings precontracted with phenylephrine (0.3 µM). In experiments involved denuded aortic rings, a relaxation ≤10% by acetylcholine indicated a satisfactory removal of endothelium and only such tissues were used for experiments.

**Effects of SIPE on isolated rat aortic rings** — Two tonic responses to phenylephrine (1 µM) or KCl (60 mM), which stabilized in 10 min, were registered. After a third response, different concentrations of SIPE (45, 90, 135, and 180 µg/ml) were added cumulatively to isolated aortic preparations. Some experiments were also conducted in which phenylephrine or KCl was added to the tissue and left for at least 30 min to observe whether the tension was maintained during the period. The effect of SIPE on the resting tone of aorta was also studied. The relaxation was measured by comparing the developed tension before and after addition of SIPE.

**Influence of muscarinic, β-adrenergic or potassium channel blockade on the vasorelaxant effect of SIPE against phenylephrine-induced contractions** — Two tonic responses to phenylephrine (1 µM), which stabilized in 10 min, were registered. Aortic rings were then incubated in presence of atropine (1 µM), propranolol (10 µM) or glibenclamide (10 µM) for 10 min and a third response to phenylephrine was obtained and SIPE (45, 90, 135, and 180 µg/ml) was added cumulatively to isolated aortic preparations. The effectiveness of the muscarinic blockade was verified by the loss of relaxing response of the preparations to acetylcholine (10 µM) in presence of atropine (1 µM). Similarly the effectiveness of the β-adrenergic blockade was verified by the loss of relaxing response of the preparations to salbutamol (10 µM) in presence of propranolol (10 µM).

**Influence of endothelium on the vasorelaxant effect of SIPE against phenylephrine-induced contractions** — In this experiment, phenylephrine-induced sustained contractions were obtained in endothelium-denuded rings or in endothelium-intact preparations in presence of NO- nitro-L-arginine methyl ester (L-NAME, 300 µM), indomethacin (10 µM) or methylene blue (10 µM). L-NAME, indomethacin or methylene blue was added 10 min before the addition of phenylephrine. After a third phenylephrine-induced contraction, SIPE (45, 90, 135, and 180 µg/ml) was added cumulatively to isolated aortic preparations. The results obtained were compared with those obtained after addition of SIPE (45, 90, 135, and 180 µg/ml) in the course of the concentration–response experiment on phenylephrine-induced contraction.

**Drugs** — SIPE was dissolved (200 mg/ml) in dimethyl sulphoxide (DMSO) and further diluted in distilled water. Indomethacin was dissolved (10 mM) in absolute ethanol and further diluted in distilled water. Stock solutions (10 mM) of all other drugs/chemicals were prepared in distilled water. Subsequent dilutions of all stock solutions were made freshly with distilled water on the day of the experiment.

**Statistical analysis** — The results were expressed as percent inhibition. The percent inhibition at each dose level was averaged and the standard deviation (SD) was calculated. Experiments were carried out in triplicate and in some cases n=6. Further, IC₅₀ values were calculated by regression analysis for each experiment and mean IC₅₀±SE was calculated along with 95% confidence intervals (CI). The difference between the mean values was tested for statistical significance using Student’s ‘t’ test (paired). A value of P < 0.005 was considered statistically significant.

**Results**

Preliminary phytochemical screening of SIPE revealed the presence of anthraquinone compounds and phenolic compounds. The TLC of SIPE revealed the development of at least 10 spots (Fig. 1). The spots of anthraquinone and phenolic compounds were identified with methanolic KOH and FeCl₃ as spraying reagents respectively. The development of magenta-red colour and blue colour indicated the presence of anthraquinone and phenolic compounds respectively. The relative concentration of these two phytoconstituents in SIPE was found to be 30.05 and 11.50% for anthraquinone and phenolic compounds respectively. The IR fingerprint of orange-yellow spot was comparable with the reported IR-bands for anthrasesamones.

SIPE up to the concentration of 90 µg/ml relaxed the resting tone of isolated rat aorta to the extent of 28.3±6.3%. SIPE up to the concentration of 180 µg/ml completely inhibited phenylephrine (1 µM)-induced contraction while it could inhibit high K⁺-induced contraction to the extent of 70.19±3.43% (Fig. 2). The IC₅₀ of SIPE on phenylephrine- or KCl-induced contraction were 77.3±3.86 (67.39–87.21,
95% CI) and 121.4±8.77 (107.5–135.4, 95% CI) respectively. The addition of excess of propranolol (10 µM) or atropine (1 µM) to the organ bath system failed to prevent the relaxation induced by SIPE on phenylephrine-induced contractions (Fig. 3a and Fig. 3b). Similarly, the addition of indomethacin (10 µM) or glibenclamide (10 µM) could not prevent the SIPE-induced relaxation in phenylephrine-induced contraction of intact aortic preparations (Fig. 3c and Fig. 3d).

The addition of excess of L-NAME (300 µM) or methylene blue (10 µM) to the organ bath system, prevented the SIPE-induced relaxation significantly to the extent of 34.26% and 25.66% respectively. However, in denuded aortic preparations where functional endothelium was removed, SIPE could elicit relaxation to a very little extent of 6.70% (Fig. 2).

**Discussion**

Preliminary experiments have indicated that the contractile response produced by phenylephrine attained a maximum within 10 min and was relatively well sustained over the subsequent 30 min. SIPE was found to relax the resting tension slightly. SIPE could inhibit phenylephrine and KCl-induced contractions of rat aorta in a concentration dependent manner in intact-aortic rings. SIPE’s vasorelaxant activity was not modified by propranolol which reveals SIPE’s actions were not mediated through β-adrenoceptors.

In intact-aortic preparations, SIPE antagonized the phenylephrine- and KCl-induced contractions in a concentration-dependent manner. It is well-established that NO is a major endothelium derived relaxing factor (EDRF), both in vivo and in vitro, and that the release of NO from endothelial cells leads to relaxation of vascular smooth muscle cells and plays a critical role in the maintenance of vascular tone. To ascertain whether the vasorelaxant activity was mediated through endothelium-dependent pathways, further experiments were carried out on phenylephrine-induced contraction in isolated rat aorta under the blockade of EDRF or absence of functional endothelium. Thus, to determine whether part of the vasorelaxant effect produced by SIPE in isolated aortic rings could be due to NO release, experiments were performed in intact aortic preparations (pre-contracted with 1 µM phenylephrine) incubated with L-NAME (300 µM), a competitive inhibitor of NO-synthase. Under these conditions, SIPE-induced vasorelaxation was attenuated significantly to the extent of 34.26%, but not abolished completely.

The stimulation of M3-muscarinic receptors produces vasodilation, despite the lack of vascular cholinergic innervation. These muscarinic receptors are located on the endothelial cells and their stimulation leads to the release of the endothelium-derived relaxing factors, mainly NO, which diffuse to adjacent vascular smooth muscle cells and cause them to relax. To determine whether SIPE-induced vasorelaxation was mediated by NO release due to the stimulation of the endothelial M3 receptors, experiments were performed in intact aortic preparations that were pre-incubated with atropine (muscarinic antagonist). In these preparations, the vasorelaxant effect of SIPE was unaffected, which allowed to rule out the participation of SIPE in the stimulation of vascular muscarinic receptors. However, atropine has strongly inhibited vasodilatation induced by 10 µM ACh (data not shown).

In order to ascertain the involvement of other endothelium-derived relaxing factors in the vasorelaxant activity of SIPE, further experiments were carried out in presence of indomethacin, a cyclooxygenase inhibitor, to verify the involvement of prostacyclin. The vasorelaxant activity of SIPE was...
not modified in aortic rings pre-treated with indomethacin. Taken together, these results indicated clearly that SIPE-induced vasorelaxation is chiefly due to the endothelium-dependent release of NO and not mediated by prostacyclin. These findings not only demonstrate that prostanoids are likely not involved in SIPE-induced vasodilatation, but also that the SIPE effect is not due to activation of muscarinic receptors, a possible mechanism in NO- and endothelium-dependent vasodilatation. Thus the vasorelaxation could be accompanied by an increase in vascular cGMP levels¹⁰-²².

The role of functional endothelium in SIPE-induced vasorelaxation was further studied in phenylephrine-induced contraction in presence of methylene blue (an inhibitor of soluble guanylate cyclase)²³ or in absence of functional endothelium. Under these conditions, SIPE-induced vasorelaxation of the pre-contracted tissues were significantly
attenuated. These data indicate that, chiefly in part, the vasorelaxant effect of SIPE was dependent on endothelium-derived relaxing factors.

Thus, the results of SIPE-induced vasorelaxation has suggested that NO is the main endothelium-derived factor involved in SIPE activity. Although in SIPE-induced vasorelaxation, NO is a major vasorelaxant, the later is a reactive species with a short half-life (in seconds), and oxidative species such as superoxide is known to inactivate NO rapidly\(^{24,25}\). Thus, the NO-mediated physiological effect is not long lasting. However, in the present investigations, SIPE exhibited prolonged vasorelaxant activity. Polyphenols and tannins are reported to scavenge superoxide\(^{26,27}\). Thus SIPE-mediated prolonged relaxant activity could be attributed to scavenging of superoxide radicals or other reactive species by anthraquinone and/or phenolic compounds present in SIPE resulting in the extended physiological half-life of NO. In another set of experiments in our laboratory, SIPE was also found to scavenge free radicals (data not shown).

The results indicated that the vasorelaxant activities of SIPE were affected in presence of L-NAME or methylene blue or in absence of functional endothelium. It thus appeared that the vasorelaxation caused by SIPE was chiefly through endothelium-dependent, NO-guanylyl cyclase pathway and little via direct action on the arterial smooth muscle. It is well known that the maintenance of smooth muscle contraction depends upon Ca\(^{2+}\) entry from extracellular fluid through voltage-operated calcium channels (VOCCs) and/or receptor-operated calcium channels (ROCCs)\(^{28,29}\). It is well known that KCl induces smooth muscle contraction through activation of VOCCs and subsequent release of calcium from the sarcoplasmic reticulum\(^{30,31}\), without changing other signal transduction systems including phosphatidylinositol turnover and calcium sensitization\(^{32}\). SIPE inhibited high potassium-induced contractions in a concentration-dependent manner. The vasorelaxant effect of SIPE in high potassium-induced contraction indicated that the activity was not mediated through opening of the potassium channels as high potassium-induced contractions cannot be antagonized by potassium channel openers\(^{33}\). This was further supported by the inhibitory activity of SIPE on phenylephrine-induced contraction in presence of excess of glibenclamide (ATP sensitive potassium channel blocker). Thus the partial/smaller extent of vasorelaxation by SIPE (higher concentration) in presence of L-NAME or methylene blue or in absence of functional endothelium might be attributed to the blockade of VOCCs.

The vasorelaxant activity could be due to the phytoconstituents in SIPE which may have activated the endothelial nitric oxide synthase (eNOS)\(^{34,35}\). One major finding of the present study is that SIPE cause NO- and cGMP-mediated potent vasorelaxation in isolated rat aortic rings that had been pre-contracted with the phenylephrine (\(\alpha_1\)-adrenergic receptor agonist) or KCl. Vasorelaxation mediated by high concentrations (i.e. >90 µg/ml) of SIPE appear to be complex, suggesting the additional involvement of an endothelium-independent vasorelaxing pathway. Although only the NO-mediated vasorelaxing mechanisms have been investigated on SIPE in this study, it cannot be ruled out that other vasorelaxing mediators may also play a role. Taken together, these results indicate that the SIPE-induced endothelium-dependent relaxation in rat aortic rings was chiefly mediated by nitric oxide, which was released from endothelial cells via endothelial nitric oxide synthase. Because the major resulting effect after KCl or phenylephrine stimulation is an increase in intracellular calcium concentration through calcium entry, it is suggested that the residual vasorelaxant effect observed after NO-synthase inhibition, endothelium removal and soluble guanylate cyclase inhibition is due to an endothelium-independent mechanism, possibly linked to L-type calcium blocking activity.

This study used TLC technique, which allowed us to separate anthraquinone and phenolic compounds in addition to other phytoconstituents according to their adsorption properties and identify their relative concentrations. The chemical composition of SIPE is complex since at least 10 spots were developed in TLC. This complexity was the main difficulty in identification of the active compounds from this fraction. Hence TLC fingerprinting of SIPE was considered as a mark of standard. However, further studies are planned to isolate the phytoconstituent responsible for the vasorelaxant activity.

In conclusion, the present studies identified the vasorelaxant activity of SIPE. Mechanistic studies suggested vasorelaxation is chiefly via endothelium-dependent pathway. Calcium channel blockade and/or related vasorelaxation mediators may play minor roles.
in the vasorelaxant activity of SIPE. These findings may be helpful in the establishment of SIPE as a potential anti hypertensive agent, elucidation of its pharmacological actions and its further development as a therapeutic agent.

Acknowledgement

Thanks are due to the All India Council for Technical Education, New Delhi, India for financial assistance and Indian Council for Medical Research for fellowship and financial support to one of the authors, (P.S.K.). The valuable suggestions of Dr. Rao V. S. V. Vadlamudi, Former Director, The Bombay College of Pharmacy are acknowledged.

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