Cardiovascular activity of the n-butanol fraction of the methanol extract of Loranthus ferrugineus Roxb.

Cardiovascular activity of the n-butanol fraction of the methanol extract of *Loranthus ferrugineus* Roxb.

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**Abstract**

We investigated the vascular responses and the blood pressure reducing effects of different fractions obtained from the methanol extract of *Loranthus ferrugineus* Roxb. (F. Loranthaceae). By means of solvent-solvent extraction, *L. ferrugineus* methanol extract (LFME) was successively fractionated with chloroform, ethyl acetate and n-butanol. The ability of these LFME fractions to relax vascular smooth muscle against phenylephrine (PE)- and KCl-induced contractions in isolated rat aortic rings was determined. In another set of experiments, LFME fractions were tested for blood pressure lowering activity in anesthetized adult male Sprague-Dawley rats (250-300 g, 14-18 weeks). The n-butanol fraction of LFME (NBF-LFME) produced a significant concentration-dependent inhibition of PE- and KCl-induced aortic ring contractions compared to other fractions. Moreover, NBF-LFME had a significantly higher relaxant effect against PE- than against high K⁺-induced contractions. In anesthetized Sprague-Dawley rats, NBF-LFME significantly lowered blood pressure in a dose-dependent manner and with a relatively longer duration of action compared to the other fractions. HPLC, UV and IR spectra suggested the presence of terpenoid constituents in both LFME and NBF-LFME. Accordingly, we conclude that NBF-LFME is the most potent fraction producing a concentration-dependent relaxation in vascular smooth muscle *in vitro* and a dose-dependent blood pressure lowering activity *in vivo*. The cardiovascular effects of NBF-LFME are most likely attributable to its terpenoid content.

**Key words:** *Loranthus ferrugineus*; n-butanol fraction; Cardiovascular effect; Aortic ring; Vasorelaxation; Terpenoid

**Introduction**

Herbs have been used as medical treatments since the beginning of civilization and some derivatives (e.g., aspirin, reserpine, and digitalis) have become mainstays of human pharmacotherapy. Herbs and related products are commonly used by patients who seek conventional health care. For cardiovascular disease, herbal treatments have been successfully utilized to treat patients with congestive heart failure, systolic hypertension, angina pectoris, atherosclerosis, cerebral insufficiency, and arrhythmia (1,2). Herbal medicine has also made many contributions to commercial drug preparations manufactured today including ephedrine from *Ephedra sinica* (ma-huang), digitoxin from *Digitalis purpurea* (foxglove), salicin (the source of aspirin) from *Salix alba* (willow bark), and reserpin from *Rauwolfia serpentina* (snakeroot), to name just a few (1). A naturally occurring β-adrenergic blocking agent with partial agonism has also been identified in a herbal remedy (3).

Continuing research is necessary to elucidate the pharmacological activities of many herbal remedies now being used to treat cardiovascular diseases. On this basis, we attempted to address this issue by investigating the cardiovascular activities of *Loranthus ferrugineus*.

*Loranthus ferrugineus* Roxb. (syn.: *Scurrula ferruginea*) from the Loranthaceae family is a hemi-parasitic shrub found on many dicotyledonous trees and attaches itself to the host tree through modified roots (4-7). In general, Loranthaceae members are commonly known as mistletoes, while in Malaysia they are widely referred to as dedalu, dalu-dalu or...
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sometimes dedalu api owing to the rusty appearance of the leaves. The natural habitat of this plant comprises Malaysia, Sumatra, India, Australia, and New Zealand. In Malaysia, preparations from L. ferrugineus have been widely used in traditional medicine for several therapeutic purposes. As a reputable home remedy, in addition to its use for the treatment of hypertension and gastrointestinal complaints, decoctions of L. ferrugineus are also taken for general health and for its gerontological effect. Leaves, fruits and flowers are the most common parts of L. ferrugineus used to treat high blood pressure, while the roots are employed for other therapeutic uses such as ulcer and cancer treatment (8).

A recent study from our laboratory (5) revealed the presence of potential vasorelaxing activity in the methanolic extract obtained from L. ferrugineus (LFME), through its ability to inhibit noradrenaline-induced contractions in isolated rat aortic rings in a non-competitive and concentration-dependent manner. The latter pharmacological effect was supported by the fact that this extract also possessed significant blood pressure lowering properties in anesthetized normotensive Sprague-Dawley rats in vivo (4,5).

On the basis of these considerations, the objective of the present pharmacological investigation was to examine the vascular effects and blood pressure lowering properties of different fractions obtained from LFME. Solvent-solvent extraction of the crude LFME was carried out to obtain different fractions from this extract, which were subsequently tested for vasorelaxation activity using isolated rat aortic ring preparations in vitro. In another set of experiments, we determined the blood pressure lowering effect of these fractions in vivo using the anesthetized Sprague-Dawley rat model.

Material and Methods

Plant materials
The fresh aerial parts (leaves, stems, twigs, flowers, and berries) of L. ferrugineus (10 kg) were collected from the main campus of Universiti Sains Malaysia (USM), Penang, Malaysia, in July 2007. The plant was authenticated by Mr. Adnan Bin Jaafar, a taxonomist from the School of Biological Sciences, USM. A voucher specimen (No. 10943) was stored in the herbarium of the same institute.

Preparation of L. ferrugineus methanol extract and its fractions
LFME was obtained as previously described (5). In brief, a Soxhlet apparatus (Schott Duran, Germany) and solvents of ascending polarity (petroleum ether, chloroform, ethyl acetate, methanol, and water) were used to obtain LFME. Subsequently, 40 g freeze-dried LFME was suspended in 350 mL distilled water and sonicated for 30 min. The mixture was then transferred to a 1-L separatory funnel (Schott Duran) and, using solvent-solvent extraction, LFME was sequentially fractionated with chloroform (4 x 300 mL), ethyl acetate (4 x 300 mL), and n-butanol (4 x 300 mL). The resulting fractions were then concentrated on a rotary evaporator (Büchi, Switzerland) under reduced pressure (-760 mmHg) and finally freeze-dried.

The yields obtained by LFME fractionation were 38.8% chloroform fraction (CF-LFME), 1.6% ethyl acetate fraction (EAF-LFME), 32.3% n-butanol fraction (NBF-LFME), and 27.3% water fraction (WF-LFME).

Drugs and solutions
Chloroform, ethyl acetate and n-butanol were purchased from Fisher Scientific (UK). For the in vitro experiments, phenylephrine (PE) was purchased from Sigma-Aldrich (Germany), while NaCl, KCl, CaCl2, 2H2O, MgSO4, KH2PO4, glucose, and NaHCO3 were purchased from R & M Chemicals, Ltd. (UK). Pentobarbitone sodium (Rhone Merieux, France) and heparin (Leo Pharmaceuticals, Denmark) for the in vivo experiments were used as commercially available injectable solutions.

For the isolated aortic ring experiment, LFME fractions, PE, and KCl were immediately dissolved in a freshly prepared Kreb’s physiological solution (KPS) of the following composition: 118.2 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 2H2O, 1.2 MgSO4, 1.2 mM KH2PO4, 11.7 mM glucose, and 25.0 mM NaHCO3.

For the in vivo protocol, CF-LFME, EAF-LFME, NBF-LFME, and WF-LFME were dissolved in isotonic saline (0.9% NaCl solution) followed by sonication. All iv boluses were administered in a volume of 200 μL. Each iv dose was followed by a 100-μL flush of saline to ensure complete delivery of the dosage.

Animals
Adult male Sprague-Dawley rats (250-300 g, 14-18 weeks) bred and provided by the USM Animal House Facility, Penang, Malaysia, were used. The rats were housed under standard environmental conditions (25°C, 60-70% humidity) on a 12-h light-dark cycle. They were fed normal commercial rat chow (Gold Coin Feed Mills Sdn Bhd, Malaysia) and water ad libitum. The rats were allowed to acclimatize in the animal transit room for a minimum of 1 week prior to being used for any experiment. All animal handling procedures and care were in accordance with the guidelines of the Animal Ethics Committee, USM, Penang, Malaysia. The number of determinations for each in vitro isolated rat aortic ring experiment was 8 while each experimental group consisted of 6 animals for the in vivo experiments.

Preparation of rat thoracic aorta rings and experimental protocol
Rats were anesthetized with pentobarbitone sodium (60 mg/kg body weight, ip) and subsequently bled and exsanguinated. A midline incision was made through the sternum to expose the thoracic aorta. The aorta was carefully isolated, freed from surrounding fat and adherent connective tissue
and cut into 3-5 mm long rings. The rings were suspended horizontally in tissue chambers containing 10 mL KPS. Special care was taken to avoid damage to the endothelium. The tissue bath solution was constantly bubbled with a mixture of 95% O₂ and 5% CO₂ (carbogen) at 37°C. All aortic rings were allowed to equilibrate under a resting tension of 1 g for 30 min. During this period, KPS was replaced every 15 min to protect against interfering metabolites (9,10) and, if needed, the tension was readjusted to 1 g. Responses were recorded isometrically via a force-displacement transducer (P23 ID Gould, Statham Instrument, UK) coupled to a Grass polygraph model 79D (Quincy, USA) (5,11).

After stabilization, different concentrations of the fractions (0.01 µg-3.0 mg/mL) were added cumulatively to isolated aortic rings precontracted with either PE (1 µM) or KCl (80 mM) and the percent drop in the maximum contractile effect of the respective agonist was determined.

Animal surgical procedure and experimental protocol

Rats were starved over a period of 12 h with free access to water. Anesthesia was induced with pentobarbitone sodium at a dose of 60 mg/kg (ip). A PP250 endotracheal cannula (Portex, UK) was inserted into the trachea to facilitate spontaneous respiration. The left jugular vein was then cannulated with PP50 tubing (Portex) to permit the administration of additional bolus injections of anesthetic (10 mg/kg in saline, iv) occasionally and the administration of bolus doses of LFME fractions. The right carotid artery was also catheterized with PP50 tubing (Portex) filled with heparinized saline (50 IU heparin per mL saline) for direct measurement of systolic and diastolic blood pressure via a pressure transducer (P23 ID Gould, Statham Instrument) connected to a Grass polygraph model 79D (Quincy). Subsequently, a small abdominal incision was made to expose and cannulate (PP10, Portex) the urinary bladder to allow free passage of urine from the kidneys. Upon completion of surgery, 2 mL normal saline was given slowly and intravenously through the jugular vein and the animal was then allowed to stabilize for 1 h before the administration of plant fractions (4,5).

Immediately after stabilization, baseline control mean arterial pressure (MAP) values were obtained, followed by the administration of increasing doses of LFME (25, 50, 100, and 200 mg/kg at 10-min intervals) and of each fraction (12.5, 25, 50, and 100 mg/kg at 10-min intervals) as bolus iv injections over 15 s. A lower dose range was used with the fractions to check the effect of the purification step on the potency of the fractions.

Chemistry

The following analytical procedures were performed on LFME and its most biologically active fraction:

High pressure liquid chromatography (HPLC) of LFME and the active fraction. The HPLC system consisted of an HP-1100 Agilent Technologies apparatus equipped with a quaternary pump, online degasser, autosampler, automatic injector, column heater, and UV detector. Chromatographic separations of the extracts were performed on a Nucleosil C-18 column (4.6 x 250 mm, 5 µm). Isocratic solvents were used with the mobile phase being a mixture of solvent A (H₂O) and solvent B (methanol) (20:80). The column temperature was maintained at 25°C, the injection volume was 10 μL and the flow rate was 0.7 mL/min.

Lyophilized LFME and the most active fraction were dissolved in methanol, sonicated for 15 min and then prepared to a concentration of 10 mg/mL with methanol. The samples were filtered through a 0.45-µm Whatman filter. The separation of the peaks in LFME and the most active fraction was achieved with a mobile phase containing methanol and deionized water on a 5 µm Nucleosil C-18 column (4.6 x 250 mm) at a flow rate of 0.7 mL/min and detection at 210 nm with a 20-min separation time.

Ultraviolet-visible (UV-Vis) spectroscopy of LFME and the active fraction. One milligram of either LFME or the active fraction was dissolved in 10 mL HPLC grade methanol and the UV spectra were measured with a Perkin Elmer Lambda 25 UV/Vis double beam spectrophotometer (USA) with HPLC grade methanol and scanned at a wavelength range of 200-600 nm.

Fourier transform infrared (FTIR) spectroscopy of LFME and the active fraction. One milligram of either LFME or the active fraction was triturated with 100 mg KBr into a fine powder. The mixtures were then transferred to a stainless steel disc of a hydraulic press and compressed to produce thin transparent pellets. Subsequently, the pellets were inserted into an FTIR spectrophotometer (Thermo Nicolet, USA) and read in the range of 4000-200 cm⁻¹.

Data analyses

All data are reported as means ± SEM. Statistical analyses were done by one- and two-way analysis of variance (ANOVA) followed by the Bonferroni/Dunnett post hoc test (SUPERANOVA, Abacus Concepts, Inc., USA). The differences between means were considered to be significant at the 5% level. The median effective concentration (EC₅₀) and maximum response of the respective agonist tested (R max) were analyzed using computer software (Prism version 5.0, GraphPad software, USA).

Results

Effects of L. ferrugineus fractions on maximum relaxation of aortic rings

PE (1 µM) and high K⁺ (80 mM) induced sustained contractions in the isolated aortic rings, reaching a maximum tension of 1.01 ± 0.08 and 1.03 ± 0.07 g, respectively. Cumulative addition of NBF-LFME to the bath produced the most significant (P < 0.05) concentration-dependent inhibition of these sustained contractions compared to other
Figure 1. Effects of fractions obtained from the *Loranthus ferrugineus* methanol extract (LFME) on the maximum relaxation response of (A) 1 µM phenylephrine (PE)-precontracted and (B) 80 mM KCl-precontracted isolated rat aortic ring preparations. Data are reported as means ± SEM (N = 8). *P < 0.05 compared to other fractions at corresponding concentrations (one-way ANOVA followed by the Bonferroni/Dunnett post hoc test). CF-LFME = chloroform fraction; EAF-LFME = ethyl acetate fraction; NBF-LFME = n-butanol fraction; WF-LFME = water fraction.
fractions obtained from LFME (Figure 1A,B). The $R_{\text{max}}$ and EC$_{50}$ values of all the fractions obtained from LFME against PE- and KCl-induced contractions are given in Table 1. The $R_{\text{max}}$ value was significantly higher (P < 0.05) for NBF-LFME against both PE- and KCl-induced aortic ring contractions in comparison to other fractions. At concentrations of 0.1-3 mg/mL, NBF-LFME caused a significantly higher (P < 0.05) relaxant effect against PE-induced than high K$^+$-induced contractions (Figure 2).

### Effects of LFME fractions on MAP in anesthetized rats

NBF-LFME at doses of 12.5-100 mg/kg, iv, produced an immediate dose-dependent hypotensive effect in anesthetized rats, which was significantly (P < 0.05) higher than that obtained with the other fractions (Figure 3A). Moreover, the blood pressure lowering effect of NBF-LFME was characterized by a significantly (P < 0.05) longer duration of action compared to other fractions (Figure 3B). Furthermore, a 50% reduction in NBF-LFME iv dose produced a blood pressure lowering effect almost equipotent to that of the crude LFME (Figure 4).

### High pressure liquid chromatography of LFME and NBF-LFME

HPLC profiles of LFME and NBF-LFME are shown in Figure 5. Following chromatographic separation, HPLC showed major peaks at 3.7 and 4.1 min in both LFME and NBF-LFME. However, an additional peak at 7.3 min was observed in LFME.

<table>
<thead>
<tr>
<th>LFME fraction</th>
<th>PE-precontracted rings</th>
<th>KCl-precontracted rings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{50}$ ($\mu$g/mL)</td>
<td>$R_{\text{max}}$ (%)</td>
</tr>
<tr>
<td>CF-LFME</td>
<td>217</td>
<td>22.4 ± 7.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>933</td>
</tr>
<tr>
<td></td>
<td>EC$_{50}$ (µg/mL)</td>
<td>$R_{\text{max}}$ (%)</td>
</tr>
<tr>
<td>EAF-LFME</td>
<td>0.7</td>
<td>15.8 ± 9.1</td>
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<tr>
<td></td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td>NBF-LFME</td>
<td>38.4</td>
<td>80.5 ± 4.8*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>726</td>
</tr>
<tr>
<td></td>
<td>EC$_{50}$ (µg/mL)</td>
<td>$R_{\text{max}}$ (%)</td>
</tr>
<tr>
<td>WF-LFME</td>
<td>290</td>
<td>53.7 ± 5.8</td>
</tr>
<tr>
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<td>8.29</td>
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</table>

EC$_{50}$ was obtained from the concentration-response curve of LFME fractions and is reported as the median concentration required to elicit a 50% reduction of the maximum contraction induced by the respective agonist. $R_{\text{max}}$ values are reported as means ± SEM of 8 determinations. *P < 0.05 vs other fractions (one-way ANOVA followed by the Bonferroni/Dunnett post hoc test). CF-LFME = chloroform fraction; EAF-LFME = ethyl acetate fraction; NBF-LFME = n-butanol fraction; WF-LFME = water fraction.

**Figure 2.** Effect of the n-butanol fraction of *Loranthus ferrugineus* methanol extract (NBF-LFME) on the maximum relaxation response of 1 µM phenylephrine (PE)-precontracted or 80 mM KCl-precontracted isolated rat aortic ring preparations. Data are reported as means ± SEM (N = 8). *P < 0.05: significant difference between the two groups (one-way ANOVA followed by the Bonferroni/Dunnett post hoc test).
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Ultraviolet-visible spectroscopy of LFME and NBF-LFME

The UV spectrum of LFME revealed a major peak at 229 nm while that of NBF-LFME showed a peak at 210 nm.

Fourier transform infrared spectroscopy of LFME and NBF-LFME

The results of FTIR spectroscopy of LFME and NBF-LFME are given in Table 2. The data clearly show the absence of a fingerprint or carbonyl region in the spectrum of both LFME and NBF-LFME.

Discussion

We recently showed that LFME produces a concentration-dependent drop in noradrenaline-induced vascular smooth muscle contraction in isolated rat aortic ring preparations *in vitro* (5) and a dose-dependent hypotensive effect *in vivo* (4,5). We then obtained evidence indicating that the cardiovascular effects of LFME are largely retained in its n-butanol fraction. NBF-LFME, unlike other fractions, produced the most significant concentration-dependent attenuation of both PE- and KCl-induced contractions of isolated rat aortic rings compared to other fractions. These findings indicated the presence of vasodilator substances in this fraction.

It is well established that the influx of external Ca^{2+} through specific Ca^{2+} channels or Ca^{2+} release from internal stores plays an important role in excitation-contraction coupling of smooth muscle. By acting on specific membrane receptors, PE induces Ca^{2+} influx through receptor-operated channels causing tonic contraction (12,13), and stimulates...
the formation of inositol 1,4,5-triphosphate (IP$_3$), which binds to and opens specific IP$_3$-receptor channels in the sarcoplasmic reticulum membrane and induces Ca$^{2+}$ release causing phasic contraction (12,14). On the other hand, the high K$^+$-induced contraction of smooth muscle is the result of an increase in Ca$^{2+}$ influx through potential-dependent Ca$^{2+}$ channels (12,15) and potential-operated Ca$^{2+}$ channel activation is also involved in PE-induced contraction. The results showed that NBF-LFME relaxed the aortic rings precontracted with high K$^+$ and PE in a concentration-dependent manner, but with a significantly greater relaxant effect against the $\alpha_1$-adrenergic receptor agonist. These results also suggested that NBF-LFME might have inhibited Ca$^{2+}$ influx through potential-dependent or

Figure 5. High performance liquid chromatographic separation of (A) the *Loranthus ferrugineus* methanol extract (LFME) and (B) its n-butanol fraction (NBF-LFME). A Nucleosil C-18 column (4.6 x 250 mm) was eluted with methanol-water at 0.7 mL/min at 25°C. The eluent is described in Methods. The samples contained 0.1 mg solid in 10-µL injection volume.
receptor-operated Ca\textsuperscript{2+} channels. While the relaxation responses to NBF-LFME against PE-induced contractions were of significantly greater magnitude compared to those observed against KCl-induced depolarization-dependent contractions, a possible involvement of the endothelium-derived factors in the vasorelaxant activity of NBF-LFME can be inferred since it is well known that the vasoconstrictor effect of α\textsubscript{1}-adrenergic receptor agonists can be modulated by endothelium-derived relaxant factors such as endothelium-derived nitric oxide and prostacyclin (16).

The results of the anesthetized rat blood pressure monitoring experiments agreed with those obtained using isolated rat aortic ring preparations. Observations from these experiments similarly showed that NBF-LFME had the most potent blood pressure lowering effect with a relatively longer duration of action compared to other fractions. These results indicated that NBF-LFME possessed the highest amount of blood pressure lowering components. It is worth mentioning that the initial purification step of LFME using solvent-solvent fractionation contributed to a significant enhancement in the potency of the most active fraction since a 50% reduction in the dose range of NBF-LFME resulted in a magnitude of MAP reduction almost similar to that observed prior to fractionation of LFME.

It is important to highlight the fact that the more polar water fraction of LFME appeared to retain some vasorelaxing and blood pressure lowering activities, which were next to those of NBF-LFME in order of potency. This observation suggests that the most active component(s) contributing to the vasorelaxant activity of the crude LFME is/are relatively polar in nature and might have been shared, to some extent, by WF-LFME and NBF-LFME. However, a higher concentration of the biologically active compound(s) was likely to be found in NBF-LFME since the most potent vasorelaxing activity was observed with this fraction.

The aforementioned results were consistent with the HPLC chemical profiling of LFME and NBF-LFME. The major peaks that appeared in the HPLC chromatogram of LFME were present in the HPLC chromatogram of NBF-LFME, while the other small peaks seen in the LFME chromatogram were absent in the NBF-LFME chromatogram. These findings indicated the effectiveness and efficiency of the fractionation step in eliminating the inactive or undesired components of LFME.

Lohézic-Le Dévéhat et al. (17) reported the presence of flavonoid constituents in \textit{L. ferrugineus}. However, in the present study, the FTIR of LFME and its active NBF-LFME fraction showed no fingerprint region as well as the absence of a carbonyl region, which could mean that the flavonoid content of the extract and its fractions may not be present in sufficient quantity to detect or may not contribute to the activity of the extract. These interpretations were confirmed when the UV spectrum of LFME showed a major peak at 229 nm while NBF-LFME showed a major peak at 210 nm. Furthermore, the HPLC profiles of LFME and NBF-LFME similarly showed peaks at 3.7 and 4.1 min. However, an additional peak at 7.3 min was observed in the case of LFME. Taken together, the results of UV, FTIR and HPLC of both LFME and NBF-LFME suggested that the major component(s) present may not have been flavonoids. However, these peaks are largely attributable to the LFME/NBF-LFME terpenoid content. The terpenoids represent a large and diverse class of naturally occurring substance that play a crucial role in traditional herbal remedies. A large body of evidence indicates that terpenoids can modulate vascular smooth muscle tone \textit{in vitro} and thus contribute to active vascular relaxation (18, 19). Numerous reports have further shown that several terpenoid constituents mediate significant hypotensive effects in normotensive conscious and anesthetized rats (19-21). These effects collectively contribute to a considerable reduction in cardiovascular morbidity and mortality. It is worth mentioning that the additional peak in LFME may have been responsible for the higher dose range required to elicit a blood pressure lowering effect equipotent to that of NBF-LFME. The identification of the active constituent(s) responsible for the observed pharmacological effects will reveal some interesting information. Studies by our research group are underway to identify the active principle(s) in this plant and to determine whether the peak at 3.7 or 4.1 min is responsible for the observed cardiovascular action of \textit{L. ferrugineus}.

In summary, NBF-LFME is the most potent fraction producing a concentration-dependent vascular smooth muscle relaxation \textit{in vitro} and a dose-dependent hypotensive action \textit{in vivo}. There is a possible involvement of endothelium-derived factors in the vasorelaxant activity of NBF-LFME. The cardiovascular effects of NBF-LFME are most likely attributable to its terpenoid content.

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