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# The uterotonic activity of compounds isolated from the supercritical fluid extract of *Ekebergia capensis*

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**Abstract**

The wood of *Ekebergia capensis* Sparrm. is used by the local Zulu community in KwaZulu-Natal Province, South Africa to facilitate childbirth. In this investigation, the uterotonic properties of extracts from this tree were evaluated using both pregnant and non-pregnant guinea pig uterine smooth muscle in vitro. The extracts were prepared using water modified supercritical carbon dioxide at 400 atm and 80°C. As samples of these extracts displayed positive results when screened for uterotonic activity, gravity column chromatography followed by NMR spectroscopy was performed in an attempt to isolate and elucidate the structures of the compounds that were present in the extract. The extract yielded five known compounds of which only two, viz. oleanonic acid and 3-*epi*oleanolic acid, displayed uterotonic activity. Receptor binding assays were subsequently performed with 3-*epi*oleanolic acid to ascertain its mode of action. Bradykinin (30 ng/100 μl) and acetylcholine (1 μg/100 μl) were used as the B2 and cholinergic receptor agonists respectively with icatibant (HOE 140) (30 ng/100 μl) and atropine (60 μ/100 μl) as their corresponding antagonists. 3-*epi*oleanolic acid was observed to mediate its effect through the cholinergic receptor. The results of this study show that two compounds from the extract of this tree possess varying degrees of agonist activity on uterine smooth muscle with minor changes in the molecular structure affecting its intrinsic activity on uterine muscle. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Supercritical fluid extraction; *Ekebergia capensis*; Uterotonic bioassay; Oleanonic acid; 3-*epi*oleanolic acid; Cholinergic receptor

## 1. Introduction

The use of traditional medicines during pregnancy currently still play an integral part in the lives of most Black South African women. This is particularly prevalent in rural areas where modern

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health care facilities are often lacking. Pregnancy is an event of great importance and many traditions and taboos are upheld to ensure a successful confinement and the birth of a healthy child [1,2]. The ingestion of plant extracts during pregnancy is common and as many as fifty-seven different plants are used [3]. Different concoctions are consumed as antenatal remedies or, more commonly, to induce or augment labour. The wood of one tree currently used by the local Zulu community to hasten the onset of labour has been identified as *Ekebergia capensis* Sparrm. belonging to the family Meliaceae [4]. This is a fairly large tree, widespread in eastern Africa from Sudan to the Cape. Decoctions made from the chopped bark are also taken as an emetic for heartburn, for coughs and other respiratory complaints while leaves are used in an infusion as a purgative parasiticide [5]. Although many chemical compounds have been isolated from this tree [6,7], there are no studies to determine the uterotonic properties of the crude extract or the isolated compounds.

The preparation of plant extracts for scientific analysis is currently usually still performed by classical liquid solvent extractions. Unfortunately, liquid solvent extractions often require several hours or even days to perform, result in dilute extracts (which must be concentrated for analysis), and may not result in quantitative recovery of target analytes. Furthermore, concerns regarding the hazardous nature of many commonly used solvents together with the cost and environmental dangers of waste solvent disposal have given support to the development of alternative sample extraction methods. The limitations of conventional methods have fueled interest in the development of supercritical fluid extraction (SFE) as an alternative to extractions using liquid solvents.

Over the last decade, SFE has been successfully applied in the food, polymer, pharmaceutical and petroleum industries for the extraction of non polar and intermediate polarity compounds from various matrices [8–12]. Supercritical fluids are advantageous for extraction due to their high diffusivities and variable solvating power. High diffusivity leads to rapid sample penetration and extraction while variable solvating power allows tuning of the extraction selectivity. SFE has therefore evolved into a powerful extraction technique as it offers many advantages for the extraction of analytes from various matrices [13–16]. Reduced extraction times as a result of the low viscosity and high diffusivity of the supercritical fluid (SF) improves mass transfer in solid and liquid matrices. SFE is often preferable to conventional methods (e.g. Soxhlet extraction). Furthermore, the low extraction temperatures required permit extraction of thermally labile compounds, and SFE does not generate large volumes of chemical waste. Due to the vast array of components found in plants, SFE is a particularly attractive technique because the solvating power of the fluid is directly related to its density which, in turn, is related to pressure and temperature. Selective extraction of analytes is therefore possible by varying the density from gas-like to liquid-like by pressure and temperature control. $CO_2$ has been the most widely used fluid for SFE primarily because it is relatively non-reactive, non-toxic, inexpensive, and has a relatively low critical temperature (32°C) and pressure (72 atm). Unfortunately, $CO_2$ is not sufficiently polar to extract highly polar components. Polar compounds therefore show limited solubility in pure $CO_2$ and in such cases either a more polar SF should be used or a modifier added. The potential of modifiers was demonstrated during the extraction of alkaloids thebaine, codeine and morphine [17]. Recently, the potential of SFE coupled directly on-line to a uterotonic bioassay was described [18] to facilitate rapid screening of plants for uterotonic activity. The plant *E. capensis* was identified, using this assay, to possess uterotonic activity.

In this study, water modified supercritical $CO_2$ was used to extract compounds from the wood of *E. capensis* followed by subsequent testing for uterotonic activity in an attempt to identify the active principles of this plant. Isolated tissues have been used effectively in medicinal plant research to obtain information on the activity and toxicity of plant extracts. Selective screening of the extract and isolated compounds was undertaken using strips of guinea pig uterine smooth

muscle in vitro, in an attempt to provide a rationale for its use during pregnancy and identify the active principles. This extraction technique was used to facilitate in rapid identification of the active compounds through the production of cleaner and more class selective uterotonically active extracts.

## 2. Materials and methods

### 2.1. Plant material

Plant material of *E. capensis* Sparrm. was obtained from the Silverglen Medicinal Plant Nursery (KwaZulu-Natal, RSA). The identity of this plant was authenticated by comparison with a reference specimen at the Natal Herbarium. The wood, after being debarked, was finely ground and left to air dry for 72 h.

### 2.2. Aqueous extraction

An aqueous extract of the wood of *E. capensis* was prepared by heating 6.0 g of milled material in 50 ml distilled water for 30 min. The resulting decoction was filtered and freeze dried.

### 2.3. Supercritical fluid extraction

Supercritical fluid extraction was performed using a home-assembled unit consisting of a Lee Scientific 501 pump (Dionex, CA) for fluid delivery and a Perkin-Elmer Sigma 3B dual FID chromatographic oven (Norwalk, CT) for temperature control. Total extractions were performed on the dry milled samples (6.0 g) at 400 atm and 80°C using SFC/SFE-grade $CO_2$ (Air Products, Allentown, PA). The samples were spiked with 2 mol % $H_2O$ and packed tightly into the extraction vessel which was then placed horizontally into the oven and the extraction performed for 50 min static period followed by a 20 min dynamic extraction period. A deactivated fused-silica capillary (30 cm × 50 μm i.d.) (SGE, Australia) was used as a linear restrictor at the extraction cell outlet and the effluent decompressed into 50 ml methanol.

### 2.4. Uterotonic bioassay

Pregnant and non-pregnant guinea pigs were obtained from the Biomedical Resource Centre, University of Durban-Westville (KwaZulu-Natal, RSA). 20% pentobarbitone (Maybaker, Port Elizabeth, RSA) was administered intramuscularly as an anaesthetic, at a dose of 0.2 g/kg and the uterus removed rapidly by midline incision into the lower abdominal cavity. The bioassay was performed by placing a 2 cm longitudinal strip of uterine muscle into a polypropylene muscle bath containing 10 ml Tyrodes solution maintained at 37°C. The lower end of the muscle was fixed to a glass capillary tube while the upper end was suspended by a thread attached to a Harvard 386 smooth muscle isotonic transducer (Harvard Apparatus Company, Inc., MA) which transformed the change in muscle length into a proportional electrical signal. This signal was recorded using an electrically driven Beckman R511A chart recorder (Beckman, Inc., IL) at a chart speed of 0.05 mm/s. A continuous supply of 95% $O_2$ and 5% $CO_2$ (MG Fedgas, Durban, RSA) was administered through the glass capillary tube at a flow rate of 60 ml/min to provide tissue oxygenation and to act as a suitable buffer. *O*-Acetylcholine hydrochloride (ACh) (BDH Chemicals, UK) was used as a standard smooth muscle stimulant at a concentration of 1 μg/100 μl. The extract and isolated compounds were dissolved in 1% DMSO solution at known concentrations and dispensed into the muscle bath using Eppendorf pipettes. Physiological fluid (Tyrodes solution) was prepared by dissolving 2.70 mmol KCl, 1.05 mmol $MgCl_2.6H_2O$, 0.40 mmol $NaH_2PO_4$, 1.80 mmol $CaCl_2$, 137 mmol NaCl, 11.90 mmol $NaHCO_3$ and 5.60 mmol D-glucose in distilled water.

### 2.5. Isolation and structural elucidation of plant components

Column chromatography was performed using silica gel 60 (0.2–0.5 mm particle size, 35–20 mesh ASTM, Merck Art 7734, Merck Chemicals, Darmstadt, Germany) with gravity elution. Thin layer chromatography (TLC) was performed using pre-coated 0.2 mm thick aluminium backed

silica gel 60 (Merck Art 5553) TLC plates. The plates were developed using ethyl acetate-methylene chloride (50:50, v/v) and the spots were visualised by spraying with a reagent comprising anisaldehyde, sulphuric acid (conc.) and methanol in the ratio 1.25:2.5:96.25. $^{1}H$ NMR and $^{13}C$ NMR spectra were recorded in $CDCl_3$ at room temperature on a Varian Gemini 300 MHz spectrometer (Varian Instruments, Palo Alto, California, USA) and all chemical shift values were recorded relative to tetramethylsilane (TMS). High resolution mass spectra were recorded on a Kratos VG 70E WRM spectrometer (VG Instruments, Cheshire, UK).

### *2.6. Identification of active components and assessment of mode of action*

The compounds isolated from the plant extract were further subjected to in vitro screening to identify those that possess uterotonic activity. An assessment of the mode of action of the active compounds was then carried out using two receptor agonists and antagonists. Bradykinin (Sigma Chemicals, St. Louis, MO) was used as the standard B2 bradykinin receptor agonist while acetylcholine (BDH Chemicals, UK) was used as the cholinergic receptor agonist. The biological activity of the compounds was assessed both before and after addition of the receptor blockers. HOE 140 (a synthetic peptide) (Sigma Chemicals) was used as the specific B2 receptor blocker while atropine (Sigma Chemicals) was used as the cholinergic receptor antagonist. Bradykinin and acetylcholine were prepared to a final concentration of 30 ng/100 μl and 1 μg/100 μl respectively while HOE 140 and atropine were made to a final concentration of 30 ng/100μl and 60 μg/100 μl respectively. Accurate doses were dispensed into the muscle bath using Eppendorf pipettes.

### *2.7. High performance liquid chromatography*

HPLC was carried out using a Hewlett Packard HP 1090 liquid chromatograph and a UV photodiode array detector (Hewlett-Packard, Waldbronn, Germany). A 1 mg quantity of the SFE extract was dissolved in 1 ml methanol and 20 μl injected onto a Bondclone-10 $C_{18}$ reverse phase column packed with 5 μm particles (300 × 3.9 mm i.d., Phenomenex, Torrance, CA). A 70 × 3.9 mm guard column packed with 5 μm C18 packing (Phenomenex) was inserted between the solvent delivery system and the column to increase column lifetime and ensure that contaminants did not interfere with the analyses. Solvents were prepared from HPLC grade reagents and Milli-$Q^{50}$ water and filtered through 0.45 μm HV organic-aqueous compatible filters (Millipore Coporation, Milford, MA). All solvents were degassed with helium prior to use. Columns were washed with and stored in methanol after each run. All injections were performed through the automatic injector. A UV photodiode array (PDA) detector was used to scan the entire UV wavelength region and 280 nm was found to be the optimum wavelength at which most of the eluting compounds were detected. A linear gradient from 100% $H_2O$ to 100% methanol was employed to separate the components over a 25 min period.

## 3. Results and discussion

### *3.1. Uterotonic bioassay*

Guinea pigs were used as they are small, cheap and easily available models for certain aspects of human reproductive endocrinology. They have spontaneous ovulations with cyclic, progesterone-secreting corpora lutea of about 14 days duration [19], their uterine motility is not influenced by progesterone [20] and the placenta is steroid-secreting [19]. An important advantage of in vitro analysis is the elimination, to a large extent, of pharmacokinetic variables. The response to an agonist is proportional to the concentration of that agonist and the density of its receptor sites. Unfortunately, in intact animals, this concentration is determined by many other factors besides the dose of the agent administered. Thus, a change in the response to a fixed dose might be brought about, not because the target organ has become more or less sensitive, but because a different concentration of the agent is

reaching the receptor site as a result of changes in pharmacokinetics. Although changes of a pharmacokinetic nature can occur in vitro, the number of variables is considerably reduced and interpretations are more direct.

ACh was used as the standard smooth muscle stimulant as this is the natural stimulant or mediator at postganglionic parasympathetic nerve endings. In certain cases a slightly higher dose of ACh was required to induce an agonistic response due to some tissue variation. A great deal of pharmacological inference is derived from the relative sensitivity of tissues to agonists. Many factors including animal variation with respect to agonist uptake mechanisms, number of viable receptors, and differences in the efficiency of stimulus-response mechanisms can cause heterogeneity in the sensitivity of tissue to agonists. The most common problem is animal maturity, which affects receptor density and the reactivity of the isolated tissue [21]. The adsorption of substances to surfaces and subsequent leaching into fresh physiological fluid (Tyrodes) was overcome by the use of a polypropylene bath as this was observed to eliminate the problem in previous experiments [22]. The isolated tissues which were not used immediately were preserved in a viable state by storage in physiological fluid over crushed ice with the continuous passage of 95% oxygen and 5% carbon dioxide.

### 3.1.1. Aqueous extract

Prior to SFE, the aqueous extract of this plant was prepared and tested. Irreversible rhythmic uterine contractions were initiated 100 sec after administration of 588 μg of the aqueous extract into the organ bath (Fig. 1). Attempts to reduce or stop these contractions by the addition of mepyramine, an antihistamine (histamine H1 receptor antagonist), was ineffective and as a result, a second strip of muscle tissue had to be prepared in order to confirm our initial findings. The second strip of muscle responded to 2 μg ACh however 12.5 μg of mepyramine was required to reduce the spontaneous contractile activity of the muscle. The muscle thereafter failed to respond adequately to up to 12 μg ACh, however when 700 μg of the extract was dispensed into the bath, a similar irreversible contractile response was initiated. Subsequent addition of antihistamine again failed to decrease the uterine contractions. Activation of H1 histamine receptors stimulates the contraction of smooth muscles in many organs such as the gut, uterus and bronchi. Such effects are readily blocked by H1 receptor antagonists [23], however the inability of mepyramine to reduce the contractile activity induced by the aqueous extract indicated that uterine muscle contraction was stimulated through another receptor or cellular process. These results nevertheless confirm that the aqueous extract of the wood of *E. capensis* is indeed uterotonic and that the active components do not act via histamine receptors. Although this experiment revealed the physiological effectiveness of this extract, water extracts are generally complex to analyse due to the vast array of extractables. This makes the subsequent clean-up and fractionation steps tedious and time consuming, and the isolation of the biological active compounds difficult. SFE is known to produce ex-

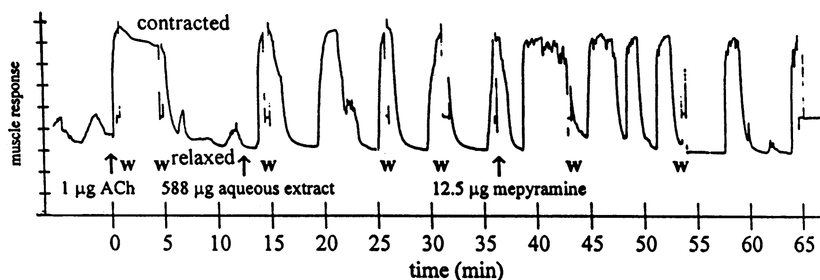


Fig. 1. Electrical recording of a pregnant guinea pig uterine smooth muscle contraction induced by 588 μg of the aqueous extract *of E. capensis.* ↑ point of injection; w = muscle wash.

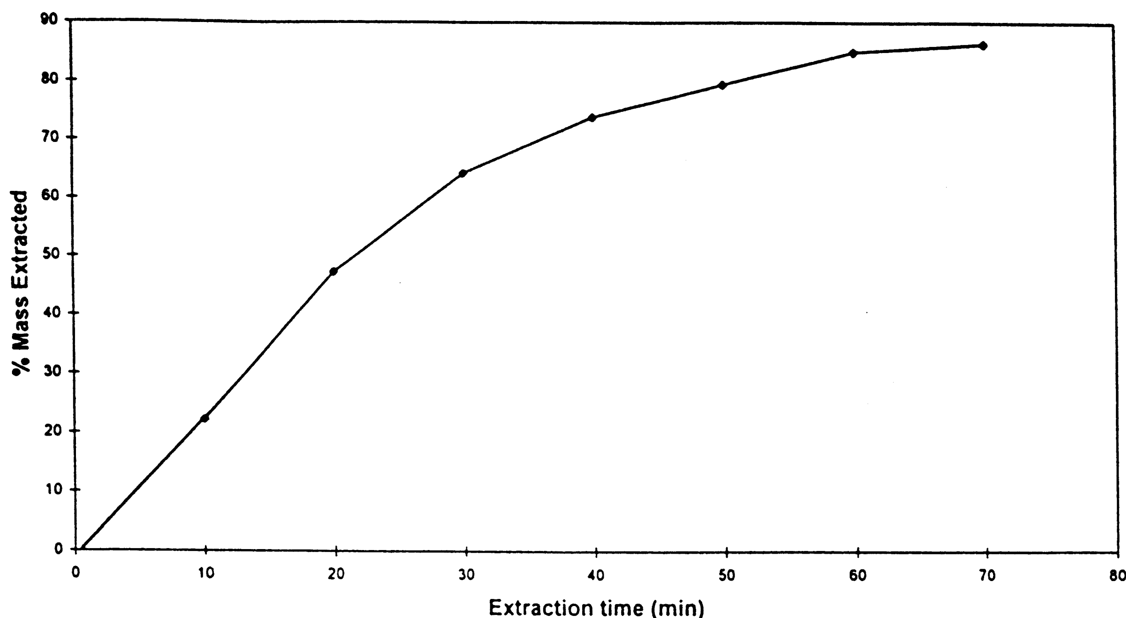


Fig. 2. Extraction curve of mass of extract from *E. capensis* as a function of extraction time (min).

tracts of lower complexity as the selectivity during extraction can be manipulated through density variation of the supercritical fluid. These observations in complexity were noted upon comparison of the TLC profile of the water extract with the SFE extract.

*3.1.2. SFE extracts*

Initial SFE extracts of *E. capensis* obtained at various densities of pure supercritical $CO_2$ were uterotonically inactive probably due to the low solubility of the active compounds and limited polarity or solvent strength of $CO_2$. The polarity of the fluid was increased by the addition of 2 mol % water directly to the plant matrix, a common practice in SFE. The addition of modifier aided the recovery of target analytes possibly due to the enhanced solubility of the analytes in the modified fluid. The addition of modifiers has also been shown to change the morphology of the substrate that is being extracted resulting in an improved extraction flux, e.g. in the extraction of caffeine from coffee [24] and also to aid in the desorption of the analytes from highly adsorptive matrices by displacing the analyte from the surface [25]. However, modifiers greatly increase the critical point of the fluid and it was therefore important that the experimental parameters were selected to maintain a single-phase region during extraction. Water is approximately 0.1% soluble (w/w) in liquid $CO_2$ at 20°C but the solubility increases to approximately 0.3% w/w at 50°C [26] indicating that the experimental conditions were indeed adequate for maintaining a single-phase. An equilibration period was also necessary to prevent the modifier from being displaced out of the extraction vessel (upon $CO_2$ entry into the cell) in order to achieve an interactive extraction. This was accommodated for by using an initial 50 min static extraction period followed by 20 min of dynamic extraction. The extractables were collected into methanol, which was later evaporated and the resulting extract weighed. The extraction conditions were optimized using the 'hot-ball' model [27] as was done in previous studies for the extraction of the limonoid, cedrelone, from *Cedrela toona* [28] and the taxol precursor, taxicin, from the English yew tree, *Taxus brevifolia* [29]. In this study, the model indicated that 85% of the extraction occurred within 60 min for the given

set of extraction conditions (Fig. 2). Thereafter the rate of extraction dropped rapidly and since extraction by supercritical fluids is never complete in finite time, it was unnecessary to continue with extractions any further.

The SFE extracts were subsequently evaluated. Fig. 3a displays the muscle contractions induced by the total SFE extract at 400 atm on a non pregnant uterus. The response to 760 μg of the extract was monitored for 260 sec. Once the crest of the contraction peak was reached, the muscle was immediately washed to facilitate rapid relaxation. Thereafter the dose of the extract was repeated however the muscle wash was performed after the muscle had spontaneously relaxed. A third dose of 3800 μg was finally administered and on each response, an increase in the area beneath the contraction peak tracing was observed indicating that the SFE extract produced a dose response uterine muscle contraction. This experiment confirmed that water modified supercritical $CO_2$ possessed the solvating power to extract the uterotonic components. When these results were compared to those obtained on a pregnant uterus, it was found that a lower dose of the extract was required to bring about a contractile response in the pregnant uterus (Fig. 3b). A plausible explanation may be increased sensitivity of the uterus as a result of hormonal changes during pregnancy. During pregnancy, the uterus is exposed to, and is altered by, the changing hormonal environment. Circulating estrogen and progesterone increase substantially due primarily to enhanced production of these hormones from the ovaries and placenta. There is also a substantial increase in the agonist receptors in the myometrium [30].

### 3.2. Isolation of compounds

Silica gel chromatography of the supercritical fluid extract led to the isolation of five compounds. From spectral evidence and chemical transformation, the compounds were identified as

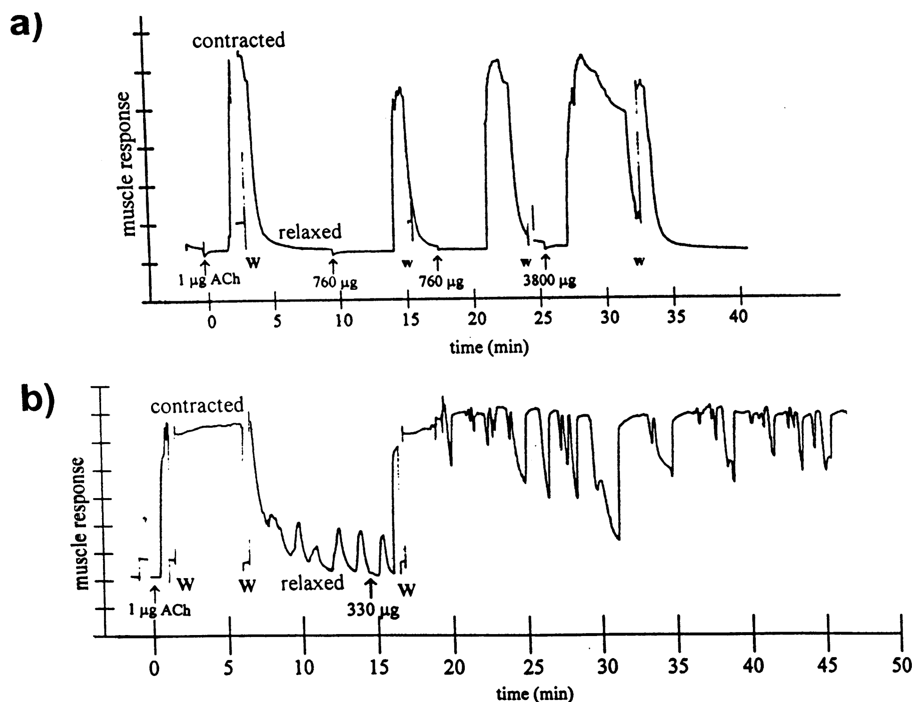


Fig. 3. Electrical recording of contractions induced by the SFE extract of *E. capensis* on a (a) non-pregnant and (b) pregnant uterus. ↑ point of injection; w = muscle wash.

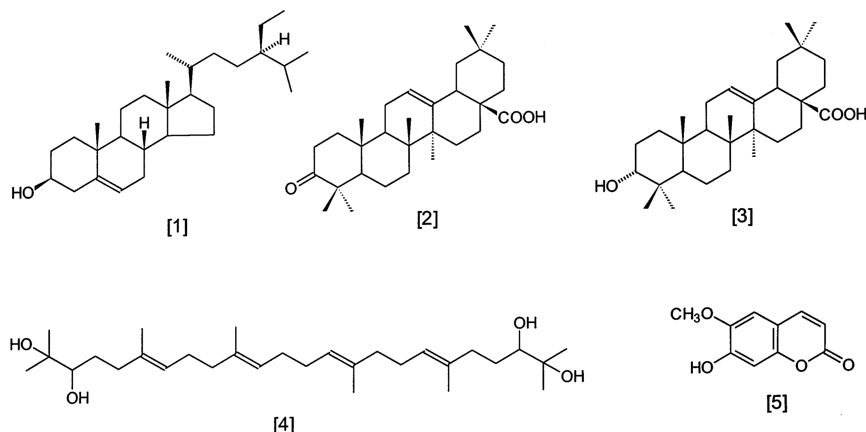


Fig. 4. The structures of five compounds isolated from the SFE extract of *E. capensis* and identified as β-sitosterol **[1]**; oleanonic acid **[2]**; 3-*epi*oleanolic acid **[3]**; 2,3,22,23-tetrahydroxy-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetra-cosatetraene **[4]** and 7-hydroxy-6-methoxycoumarin **[5]**.

β-sitosterol **[1]**; oleanonic acid **[2]**; 3-*epi*oleanolic acid **[3]**; 2,3,22,23-tetrahydroxy-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetra-cosatetraene **[4]** and 7-hydroxy-6-methoxycoumarin **[5]** the structures of which are given below (Fig. 4).

### 3.3. In vitro screening of isolated compounds

All five compounds isolated from *E. capensis* were subjected to further uterotonic bioassays to identify those responsible for uterine muscle contraction. All the compounds except **[5]** 7-hydroxy-6-methoxycoumarin showed limited solubility in 0.9% saline but dissolved readily in 1% DMSO solution. The muscle did not show any adverse effects to 1% DMSO and compounds **[2]** oleanonic acid and **[3]** 3-*epi*oleanolic acid were observed to induce uterine muscle contractions.

#### 3.3.1. Oleanonic acid [2]

Oleanonic acid was dissolved in a 1% DMSO solution at a concentration of 1.83 μg/μl. Following an agonistic response to 5 μg ACh, the muscle was observed to develop spontaneous contractions that were monitored over a 15 min period (Fig. 5). Oleanonic acid was found to elicit a muscle response after five cumulative additions of 366 μg quantities of this compound to the organ bath. The contractile activity induced by oleanonic acid varied in amplitude, with each contractile pulse lasting approximately 40 s. Subsequent addition of ACh produced a larger response than the initial 5 μg ACh indicating that oleanonic acid may have sensitised the cholinergic receptors thereby increasing the agonistic response to ACh.

#### 3.3.2. 3-epioleanolic acid [3]

3-*epi*oleanolic acid [3] was dissolved in a 1% DMSO solution at a concentration of 1.77 μg/μl. 10 μg ACh was administered to produce a muscle response followed by the addition of 354 μg quantities of 3-*epi*oleanolic acid to the organ bath (Fig. 6). Apart from the spontaneous contractions of the muscle, a cumulative dose of 708 μg was observed to increase the amplitude of the contractions in the spontaneously contracting uterus. Further additions of 354 μg quantities of this compound were found to further increase the amplitude of the contractions in a concentration-dependent manner. The muscle was thereafter washed and a subsequent addition of 10 μg ACh showed an increased ACh response once again.

Unlike oleanonic acid, this compound displayed efficacy at much lower doses. An increase in both the tonicity and frequency of contractions was also observed. The only difference between these two compounds was the presence of a keto

group at carbon 3 for oleanonic acid and the α-hydroxy group for 3-*epi*oleanolic acid. These minor changes in the molecule can affect its intrinsic activity on the muscle [31]. 3-*epi*oleanolic acid was found to induce muscle contraction at lower doses and within a shorter time period than oleanonic acid. This may have resulted from the ability of the 3-*epi*-compound to reach the active site of the receptor or to be metabolised more readily than oleanonic acid. The longer time period necessary for oleanonic acid to induce activity may also be due to a longer diffusion period required for the compound to enter the receptor compartment. The dissolution of a drug from the point of injection into a well mixed organ bath occurs relatively rapidly, however the diffusion coefficient of drugs in tissues is slower than in free solution [32]. This is due to the longer path that a drug must take through a tissue to accommodate the numerous obstructions in the morphological organization of the muscle. One factor thought to be responsible for the difference in diffusion rates is tissue thickness. Furthermore, physicochemical properties, such as electronegativity, polarizability, bond angles, van der Waal's radii, number of substituents and charge of the atom can greatly influence the physicochemical characteristics of the molecule. Drug molecules, in turn, exert their effect by influencing receptor sites through their physicochemical properties. It follows, therefore, that alteration of a group in a molecule will change the physicochemical properties of the molecule and thereby the biological response to it. The other compounds that were isolated from this plant were also subjected to bioassays however these were found to be inactive as there was no indication of uterine muscle stimulation.

### *3.4. Assessment of mode of action of 3-epioleanolic acid through receptor binding assays*

In order to determine the receptor/s through which this compound mediated its effect, use was made of two receptor agonists and antagonists. Two receptors that can be involved in smooth muscle contraction are the bradykinin B2 receptor and the muscarinic cholinergic receptor. Bradykinin was used as the standard B2 receptor agonist while acetylcholine was used as the cholinergic receptor agonist. It is well accepted that bradykinin can contract or dilate vascular smooth muscle [33] depending on (a) the type of kinin receptor, (b) the type and potency of kininases present on endothelial cells and (c) the ability to generate nitric oxide (NO) also known as endothelium derived relaxing factor (EDRF) [34]. The cholinergic receptors are also responsible for stimulating contractions of smooth muscles. The biological activity of the compounds were as-

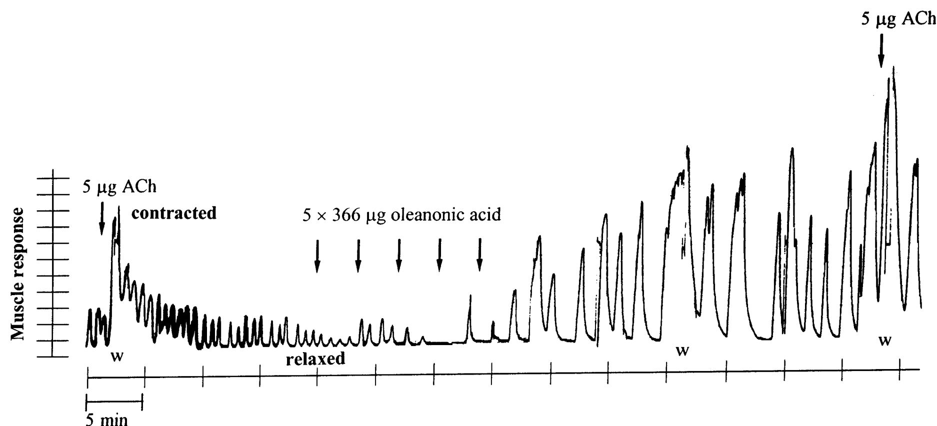


Fig. 5. Electrical recording of contractions induced by oleanonic acid on a non-pregnant uterus. ↓ point of injection; w = muscle wash.

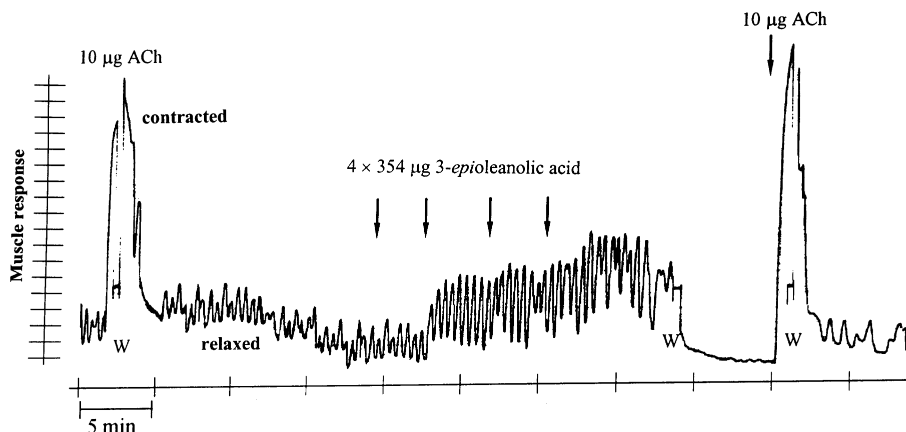


Fig. 6. Electrical recording of contractions induced by 3-*epi*oleanolic acid on a non-pregnant uterus. ↓ point of injection; w = muscle wash.

sessed both before and after addition of the receptor blockers. HOE 140 (a peptide analogue) (Sigma Chemicals) was used as the specific B2 receptor blocker while atropine (Sigma Chemicals) was used as the cholinergic receptor antagonist as it blocks postganglionic cholinergic receptors by binding to them and preventing access of acetylcholine.

Bradykinin (15 ng) was administered to the uterine muscle to elicit a maximum response (Fig. 7). Thereafter 500 μg of HOE 140 was added to the muscle bath to block the B2 receptors. Bradykinin was added once again to establish whether the B2 receptors were blocked. Failure of a muscle contraction indicated that B2 receptors were blocked as bradykinin did not produce a muscle response. 3-*epi*oleanolic acid was thereafter added to the muscle bath. A dose of 354 μg induced a maximum response indicating that this compound did not mediate its response through the B2 receptors as the muscle still contracted even after blocking off the bradykinin receptors. A subsequent addition of 1 μg ACh elicited a response and this showed the specificity of the B2 receptor blocker in that, although it blocked the B2 receptor, it did not block the cholinergic receptor. Thirty micrograms atropine was thereafter dispensed into the organ bath to block off the

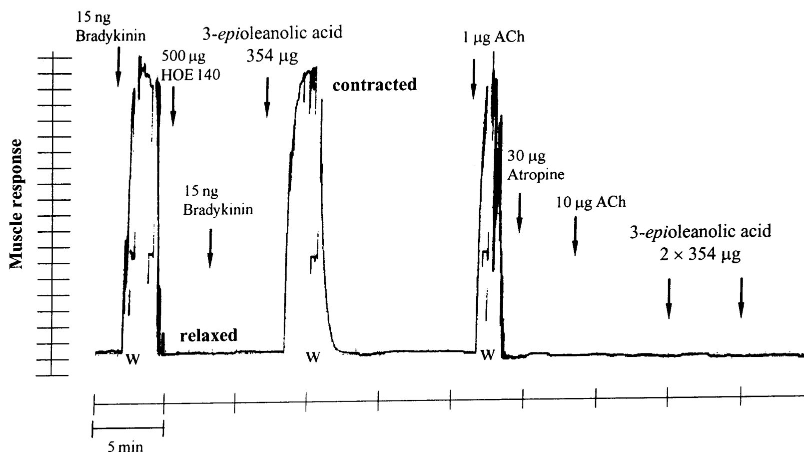


Fig. 7. Receptor binding assay of 3-*epi*oleanolic acid. ↓ point of injection; w = muscle wash.

cholinergic receptors. To confirm this, a tenfold increase of ACh was added to the bath and this failed to produce an agonistic response. Subsequent addition of two 354 μg doses of 3-*epi*-oleanolic did not initiate a muscle response indicating that the compound mediated its effect through the cholinergic receptor since blocking the receptor failed to produce a muscle response.

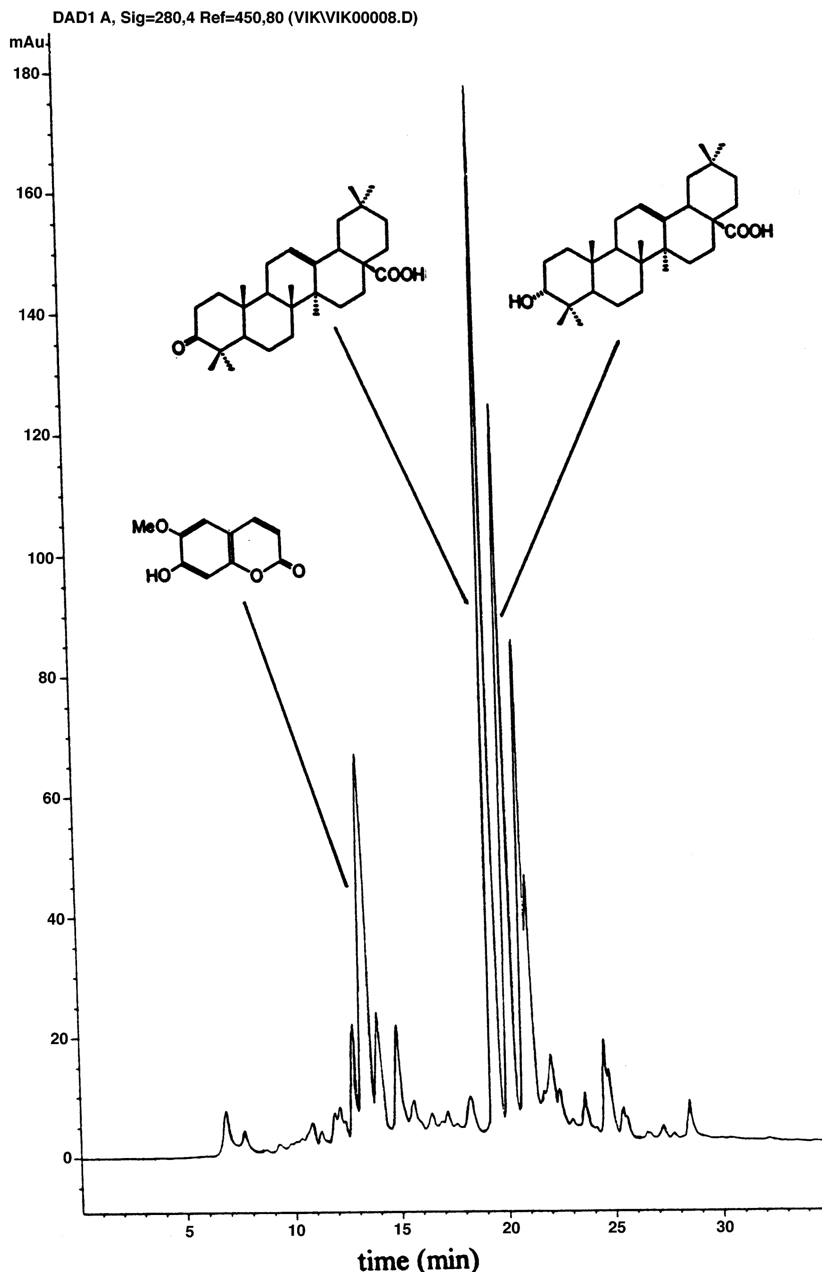


Fig. 8. Reversed-phase HPLC chromatogram of SFE extract of *E. capensis* obtained at 400 atm and 80°C. Conditions: Bondclone-10 $C_{18}$ reversed phase column; gradient elution; column temperature 40 °C; UV detection at 280 nm.

### 3.5. Chromatographic analysis

The quality control of this extract is a general requirement and is necessary if it is to fill the need for a cheap and reliable medicine. It is well known that plants synthesise a large variety of chemical compounds and variation in the concentration of the active constituents with topography, season, as well as climatic and ecological conditions is known to occur. This can result in the co-occurrence of undesirable compounds causing antagonistic, synergistic, or other undesirable, and possibly unpredictable, modulations of the bioactivity. Reversed-phase HPLC is a widely accepted, well-established and reliable analytical technique, for which numerous applications in the field of natural products have been developed. The technique was applied to this plant extract so that the chromatogram obtained can be used for monitoring variations that may occur.

Fig. 8 shows the HPLC separation of the *E. capensis* extract by gradient elution. The components were eluted within 30 min and the identity of the peaks were confirmed by injecting the pure compounds that were isolated and further confirmation was obtained by comparison of the UV spectrum on the photodiode array detector. The compound 7-hydroxy-6-methoxycoumarin commonly known as scopoletin, being more polar, eluted first from the column while oleanonic acid and 3-*epi*oleanolic acid eluted at higher methanol concentrations. Scopoletin concentrations need to be carefully monitored as it is a known antispasmodic agent and may have a relaxant effect on uterine muscle. A related compound, angelicin, previously isolated from *Heracleum thomsoni* (Apiaceae) produced a relaxant effect on a wide variety of smooth muscle preparations from various species [35].

## 4. Conclusion

This study has provided further scientific insight into the chemical constituents of *E. capensis* and has successfully validated its uterotonic properties hence providing a rationale for its use during pregnancy. Supercritical fluid extraction was found to be a simple and effective method of extracting the uterotonic components of which one was observed to mediate its effect through the cholinergic receptors. The chromatographic identification of some of the components of this extract may be used to screen physiological fluids for these compounds hence identifying its use clinically without having to rely on patient's information.

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