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DOI: 10.1515/folmed-2017-0047

ORIGINAL ARTICLE, PHARMACY

Spermicidal Constituents of Ethanolic Extract of *Sacoglottis gabonensis* Stem Bark

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Received: 05 July 2016
Accepted: 22 March 2017
Published Online: 09 May 2017
Published: 22 Dec 2017

Key words: sperm, immobilization, compound, *Sacoglottis gabonensis*

Citation: Alade GO, Moody JO, Awotona OR, Adesanya SA, Lai D, Proksch P. Spermicidal constituents of ethanolic extract of *Sacoglottis gabonensis* stem bark. Folia Medica 2017;59(4):437-42. doi: 10.1515/folmed-2017-0047

Aim: To isolate the spermicidal constituents of *Sacoglottis gabonensis*.

Materials and methods: The ethanolic extract with partitioned fractions of *Sacoglottis gabonensis* stem bark were subjected to sperm immobilization assay. The most active EtOAc fraction was further purified by column and Semi-Preparative High Performance Liquid Chromatography to give compounds which were characterized by spectroscopic methods (UV, LC/MS, and NMR). The compound(s) was also tested for sperm immobilization activity.

Results: The ethanolic extract showed 100% significant ($p < 0.05$) sperm immobilization activity at a concentration of 30 mg/mL at 20 s compared to both negative and positive controls. The most active ethyl acetate fraction yielded methyl 3,5-dihydroxy-4-methoxybenzoate, eriodictyol and bergenin. Bergenin had 100% sperm immobilization activity at 20 mg/mL in 60 s which was significant ($p < 0.05$) also when compared to the positive and negative control while methyl 3,5-dihydroxy-4-methoxybenzoate, eriodictyol were not active.

Conclusion: The active spermicidal constituent in *Sacoglottis gabonensis* stem bark extract is bergenin. However, methyl 3,5-dihydroxy-4-methoxybenzoate and eriodictyol showed no activity. This plant is known for its aphrodisiac action; hence, caution may have to be exercised in its use because of its spermicidal effect.

BACKGROUND

Fertility control is an option in human population control. It is a global and national public concern.[1] The majority of the currently available methods of fertility control are for women, participation of the male counterpart is poor.[2] Some of the safe and effective male contraceptives currently known are condoms and vasectomy.[3,4] Recently efforts are however, being made to explore the hidden wealth of medicinal plants for contraceptive use in males.[5]

In a preliminary work, *Sacoglottis gabonensis* stem bark has been previously reported for its sperm reducing potential in male rats with activity resident in the ethyl acetate fraction.[6]

AIM

This study aimed at isolating the compound(s) responsible for this activity.

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURES

^{1}H, ^{13}C and 2D NMR were recorded in deuterized solvents on Bruker DRX500 or AVANCE DMX 600 NMR spectrometers. Mass spectra were measured on a LC-MS Agilent 1100 series coupled with Thermoquest LCQ Deca XP (Finnigan). HPLC analysis was carried out on a Dionex UltiMate 3000 HPLC system (Thermo scientific) coupled to a photodiode array detector (DAD-3000RS). Routine detection

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was performed at 235, 254, 280, and 340 nm. The separation column (125×4 mm, L×i.d.) was prefilled with Eurospher-10 C18 (Knauer, Germany), and the following gradient was used (MeOH/0.1% formic acid in water); 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100% MeOH with a flow rate of 1.0 mL/min. HPLC separation was performed on a semi-preparative HPLC system of Lachrom-Merck Hitachi (Pump L7100 and UV detector L7400). The separation column (300×8 mm) was prefilled with Europhere 100-C18 (Knauer, Germany) using a flow rate of 5.0 mL/min. Column chromatography was performed using Merck MN Silica gel 60 M (0.04–0.063 mm), Diaion HP20 (Mitsubishi Chemicals) or Sephadex LH20 (GE HealthCare) as stationary phases. For TLC analyses pre-coated Silica Gel 60 F254 plates (Merck) were used followed by detection under UV at 254 and 366 nm or after spraying with anisaldehyde reagent. All solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements.

Plant material

The stem bark of *Sacoglottis gabonensis* was collected and identified by Dr. A.T Oladele of the Department of Forestry and Wildlife Sciences, University of Port Harcourt, Rivers State of Nigeria. A voucher specimen (FHI109851) was deposited at the Herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria.

Extraction and isolation

The dried powder stem bark (500 g) of *S. gabonensis* was extracted with 80% cold ethanol and the extract was evaporated to give a brown residue (25 g). The suspension of the extract in nanopure water was subjected to solvent-solvent partitioning in a separating funnel with n-hexane (400 mL x 3), ethyl acetate (400 mL x 3), n-butanol (500 mL x 1), successively, and the various partitioned fractions were concentrated to dryness *in vacuo* using a rotary evaporator to obtain 1.3341 g of n-hexane, 7.331 g ethyl acetate, 8.5091 g of n-butanol and 5.0988 g of aqueous fractions, respectively. The fractions were monitored in HPLC coupled to an UV detector. 5.0 g of the ethyl acetate fraction was dissolved in dichloromethane and adsorbed onto silica gel. This was allowed to dry and chromatographed on silica gel under vacuum using a column 30 cm in height and 6 cm inner diameter with hexane containing increasing percentages (up to 10%) of ethyl acetate, subsequently dichloromethane containing increasing percentages (up to 10%) of methanol as eluent and each collected fraction was 500 mL. Each fraction was collected, concentrated and monitored by HPLC. Eighty mg of fraction 2 was dissolved in a mixture of dichloromethane/methanol (50%) and chromatographed over Sephadex LH-20 using an open column (50 cm×4 cm) and dichloromethane/methanol (50%) as the eluent. The fraction was collected every 15 minutes using a fraction collector at a flow rate of 10 drops per second. One hundred and ten fractions were collected and pooled together with the aid of thin layer chromatography to get a total of seven fractions (1-7, respectively). Fractions 2, 4 and 6 were chosen for further purification. Due to low yields, fractions 1, 3, 5 and 7 couldn't be purified further. Each of the pooled fractions was dried using a rotary evaporator and also monitored by HPLC. Fraction 2 was finally purified using semi preparative HPLC to afford Compound 1. Fraction 4 was similarly processed using semi preparative HPLC to afford Compound 2. Fraction 6 was chromatographed on an open column (50 cm×4 cm) using HP-20 with methanol containing increasing percentages (10%) of water as the eluent to afford a pure compound which came out as sub-fraction 3 (methanol : water; 2:8) and this afforded compound 3.

Sperm immobilization assay

Healthy adult male albino rats were used for the experiments. The spermatozoa were obtained from the caudal epididymis excised from healthy adult rats after autopsy. The spermatozoa were collected into saline solution and adjusted to the concentration of 40-60×10^6 sperm/mL and incubated in a water bath at 37°C. *In vitro* spermicidal activity of various concentrations of the crude extract/fractions and compounds of *Sacoglottis gabonensis* was performed with each dilution, following the modified method of Sander and Cramer[7] and the standard recommended by the WHO[8]. A stock solution of 120 mg/mL of the extract and the various fractions/compounds was separately made and serial dilutions were made for the different concentrations to be employed. Sperm suspension (0.5 mL) was mixed with 0.5 mL of the different concentrations of the extract/fractions/compounds (7.5, 15, 30, 60 and 120 mg/mL) to evaluate the spermicidal activity. The two fluids were thoroughly mixed. Within 20, 30, 60, 120 and 180 seconds of mixing, a drop of the mixture was immediately placed on warm slides and microscopically examined under 100x to observe the

motility of the spermatozoa. A mixture of 0.5 mL of sperm suspension and 0.5 mL of normal saline and cotton seed oil served as negative and positive controls, respectively. Each experiment was carried out in ten (10) replicates. Values were represented as mean ± standard error of mean (SEM). One-way ANOVA with Tukey –Kramer Multiple Comparison Test was performed using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. P values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Spermicidal activity

From the result **(Table 1)**, *S. gabonensis* stem bark had 100% spermicidal activity at 120 mg/mL in 20 s. This corroborates the 50% significant decrease in sperm count at 30 mg/kg *in vivo* in male rats.[6]

Table 1. In vitro sperm immobilization screening of *Sacoglottis gabonensis*, fractions and compound

			Spermicidal Activity / %				
S/No.	**Plant extract / fractions / compounds**	**Concentration (mg/mL)**	**20 s.**	**30 s.**	**60 s**	**120 s**	**180 s**
1	*Sacoglottis gabonensis* extract	7.5	74.50 ± 2.63 *a,b	75.00 ± 1.05 *a,b	74.70 ± 3.06 *a,b	76.50 ± 3.87* a,b	74.40 ± 3.69* a,b
		15	75.60 ± 4.79 *a,b	74.40 ± 2.68 *a,b	76.50 ± 6.89* a,b	76.40 ± 4.55* a,b	74.00 ± 2.84* a,b
		30	100.00 ± 0.00*a,b	100.00 ± 0.00 *a,b	100.00 ± 0.00 *a,b	100.00 ± 0.00*a,b	100.00 ± 0.00* a,b
		60	100.00 ± 0.00*a,b	100.00 ± 0.00 *a,b	100.00 ± 0.00 *a,b	100.00 ± 0.00 a,b	100.00 ± 0.00* a,b
		120	100.00 ± 0.00 *a,b	100.00 ± 0.00 *a,b	100.00 ± 0.00 *a,b	100.00 ± 0.00 *a,b	100.00 ± 0.00* a,b
2	*S. gabonensis* hexane fraction	7.5	0.00	0.00	0.00	0.00	20.00 ± 2.36* a,b
		15	0.00	0.00	0.00	0.00	19.50 ± 2.84* a,b
		30	0.00	0.00	0.00	0.00	19.50 ± 4.38* a,b
		60	0.00	0.00	0.00	0.00	21.00 ± 3.16* a,b
		120	0.00	0.00	0.00	0.00	19.50 ± 1.58* a,b
3	*S. gabonensis* ethyl acetate fraction	7.5	0.00	0.00	0.00	0.00	40.00 ± 3.33* a,b
		15	0.00	0.00	0.00	0.00	39.50 ± 3.69* a,b
		30	0.00	20.00 ± 2.33* a,b	60.00 ± 2.36* a,b	59.50 ± 2.84 *a,b	100.00 ± 0.00* a,b
		60	19.50 ± 2.41 *a,b	20.00 ± 2.93 *a,b	59.00 ± 2.12* a,b	60.00 ± 3.33 *a,b	100.00 ± 0.00* a,b
		120	20.50 ± 2.40 *a,b	38.50 ±2.42*a,b	60.50 ± 4.38*a,b	10.00 ± 0.00 * a,b	100.00 ± 0.00* a,b
4	*S. gabonensis* butanol fraction	7.5	0.00	0.00	0.00	0.00	0.00
		15	0.00	0.00	0.00	0.00	0.00
		30	0.00	0.00	0.00	0.00	10.50 ± 1.58*a,b
		60	0.00	0.00	0.00	0.00	9.50 ± 1.58* a,b
		120	0.00	0.00	0.00	0.00	11.00 ± 3.16* a,b
5	*S. gabonensis* aqueous fraction	7.5	0.00	0.00	0.00	0.00	0.00
		15	0.00	0.00	0.00	0.00	0.00
		30	0.00	0.00	0.00	0.00	0.00
		60	0.00	0.00	0.00	0.00	0.00
		120	0.00	0.00	0.00	0.00	0.00
6	Methyl 3,5-dihydroxy-4-methoxybenzoate (Compound 1)	7.5	0.00	0.00	0.00	0.00	0.00
		20	0.00	0.00	0.00	0.00	0.00
7	Eriodictyol (Compound 2)	7.5	0.00	0.00	0.00	0.00	0.00
		20	0.00	0.00	0.00	0.00	0.00
8	Bergenin (Compound 3)	7.5	0.00	0.00	60.50 ± 3.69* a,b	61.00 ± 3.94* a,b	61.00 ± 3.94* a,b
		20	0.00	0.00	100.00 ± 0.00	100 ± 0.00* a,b	100.00 ± 0.00* a,b
9	Normal saline		0.00	0.00	0.00	0.00	0.00.
10	Cotton seed oil	7.5	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Data expressed as mean ± SEM (n=10), *p<0.05 (*a, when compared with normal saline and *b, when compared to cotton seed oil).

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From a previous report, it was found that activity was retained in the ethyl acetate fraction[6] as was also confirmed in the *in vitro* spermicidal result. The isolated compound, bergenin showed 100% spermicidal activity at 20 mg/mL concentration in 60 seconds while methyl 3,5-dihydroxy-4-methoxybenzoate and eriodictyol had no activity.

Compound 1 was isolated from *Sacoglottis gabonensis* as a yellow solid and identified as methyl 3,5-dihydroxy-4-methoxybenzoate **(Fig. 1)**, with UV λ_{max}^{MeOH} 260.5 and 298 nm. The molecular formula was established as $C_9H_{10}O_5$ based on spectral data obtained by ESI-MS. Positive and negative ESI-MS showed pseudo-molecular ion peaks at *m/z* (% intensity) 199.0 $[M+H]^+$ (100) (base peak) and 197.1 $[M-H]^-$ (100) (base peak), respectively, indicating a molecular mass of 198 g/mol. This was further validated by the presence of the molecular ion peak at *m/z* (% intensity) 394.8 $[2M+H]^-$ (82) in the negative ESI-MS. The ^{1}H NMR (CD_3OD): δ (ppm) 7.02 (2H, s, H-2, H-6), 3.86 (s, $-OCH_3$), 3.83 (s, $-OCOCH_3$). Comparison with literature data showed that it is methyl 3,5-dihydroxy-4-methoxybenzoate which was previously isolated from the flowers of *Tamarix nilotica*.[9,10]

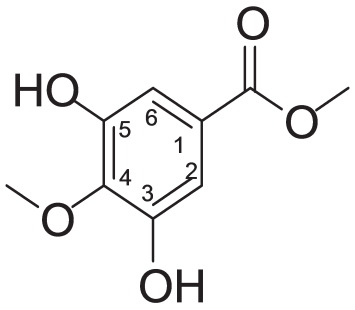

Figure 1. Methyl 3,5-dihydroxy-4-methoxybenzoate.

Compound 2 was isolated from *Sacoglottis gabonensis* as a light yellowish white solid and identified as eriodictyol **(Fig. 2)** with UV λ_{max}^{MeOH} 288 nm. The molecular formula was established as $C_{15}H_{12}O_6$ based on spectra data obtained by ESI-MS. Positive and negative ESI-MS showed pseudo-molecular ion peaks at *m/z* (% intensity) 287.2 $[M-H]^-$ (83) (base peak) and 289.2 $[M+H]^+$ (100) (base peak), respectively, indicating a molecular mass of 288 g/mol. ^{1}H NMR (CD_3OD, 500MHz): δ (ppm) 2.69 (H-3a, dd, J=3.0, 17.0 Hz), 3.06 (H-3b, dd, J=12.8, 17.0 Hz), 5.27 (H-2, d, J=12.8 Hz), 5.87 (H-6, H-8, m), 6.79 (H-5', H-6', 2H, s) and 6.91 (H-2', s). SBE4D was identified as eriodictyol by comparison with literature data.[11]

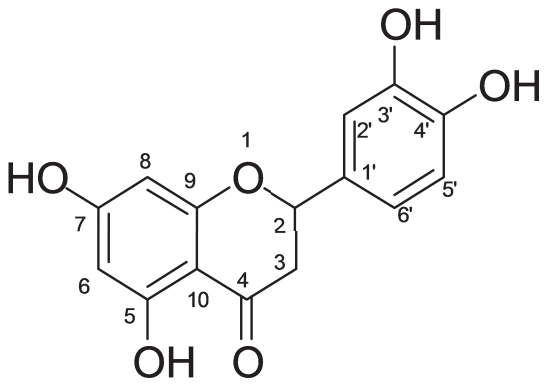

Figure 2. Eriodictyol.

Compound 3 was isolated from *Sacoglottis gabonensis* as a white crystalline solid and identified as Bergenin **(Fig. 3)**, with UV λ_{max}^{MeOH} 230, 288, and 328 nm. The molecular formula was established as $C_{14}H_{16}O_9$ based on spectral data obtained by ESI-MS. Positive and negative ESI-MS showed pseudo-molecular ion peaks at *m/z* (% intensity) 329.0 $[M+H]^+$ (100) (base peak) and 327.2 $[M-H]^-$ (100) (base peak), respectively, indicating a molecular mass of 328 g/mol. This was further validated by the presence of the molecular ion peak at *m/z* (% intensity) 654.9 $[2M+H]^-$ (20). The ^{1}H NMR (CD_3OD, 500 MHz): δ (ppm) 7.07 (H-4, s), 4.95 (H-9, d, J=10.5 Hz), 3.68 (2H, H_2-16, m), 3.90 (3H, s, 6-OCH_3), 3.44 (H-12, t, J=9.0 Hz), 3.81 (H-13, t, J=9.0 Hz), 4.05 (2H, m, H-11, H-14). These values were consistent with those already reported for bergenin.[12,13]

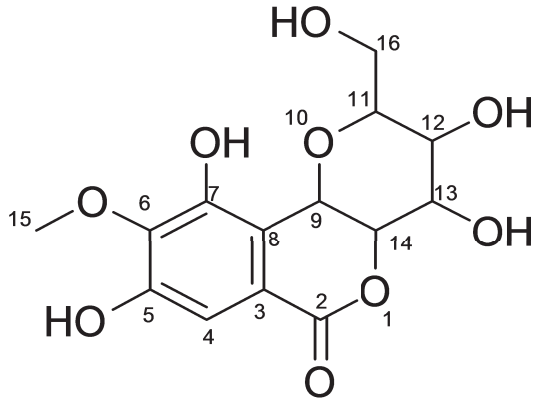

Figure 3. Bergenin.

CONCLUSION

The active spermicidal constituent in *Sacoglottis gabonensis* stem bark extract is bergenin. However,

methyl 3,5-dihydroxy-4-methoxybenzoate and eriodictyol showed no activity. This plant is known for its aphrodisiac action; hence, caution may have to be exercised in its use because of its spermicidal effect.

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Спермицидные ингредиенты экстракта этанола из коры стебля *Sacoglottis gabonensis*

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Дата получения: 05 июля 2016
Дата приемки: 22 марта 2017
Дата онлайн публикации: 09 мая 2017
Дата публикации: 22 декабря 2017

Ключевые слова: сперма, иммобилизация, ингредиент, Sacoglottis gabonensis

Образец цитирования: Alade GO, Moody JO, Awotona OR, Adesanya SA, Lai D, Proksch P. Spermicidal constituents of ethanolic extract of *Sacoglottis gabonensis* stem bark. Folia Medica 2017;59(4):437-42. doi: 10.1515/folmed-2017-0047.

Цель: Целью настоящего исследования является изолирование спермицидных ингредиентов *Sacoglottis gabonensis*.

Материалы и методы: Экстракт этанола с измельчёнными частицами коры стебля *Sacoglottis gabonensis* был исследован на предмет установления иммобилизации спермы. Наиболее активная фракция этилацетата была дополнительно очищена с применением колоночной и полупрепаративной высокоэффективной жидкостной хроматографии, с целью изолирования ингредиентов, подлежащих характеристике спектроскопскими методами (UV, LC/MS, и NMR). Ингредиент был исследован на предмет установления активности иммобилизации спермы.

Результаты: Экстракт этанола проявил 100 % активность иммобилизации спермы ($p < 0.05$) при концентрации 30 мг/мл. при 20с по сравнению как с положительными, так и с отрицательными контролями. Наиболее активной фракцией этилацетата является метил 3,5-дигидрокси-4-метоксибензоат, эриодиктиол и бергенин. Бергенин проявил 100 % активность иммобилизации спермы при 20 мг/мл при 60с, что является значимым ($p < 0.05$) также при сопоставлении с положительными и отрицательными контролями, в отличие от метил 3,5-дигидрокси-4-метоксибензоата и эриодиктиола, которые не показали активности.

Заключение: Активным спермицидным ингредиентом коры стебля *Sacoglottis gabonensis* является бергенин. Независимо от этого, метил 3,5-дигидрокси-4-метоксибензоат и изодиадиол не проявили активности. Данное растение известно как афродизиак и ввиду наличия спермицидных свойств надо проявлять осторожность при его применении.