Effects of *Kigelia africana* (Lam.) Benth. fruits extract on the development and maturation of the reproductive system in immature male rats

Veronica Micheli[a], Rokia Sanogo[b] Maria Alessandra Mobilia[c] and Francesco Occhiuto[d*]

[a]*Foundation A. Imbesi, University of Messina. Vill. SS. Annunziata 98168 Messina, Italy*
[b]*Department of Traditional Medicine, University of Bamako, B.P. 1746 Bamako, Mali, Africa*
[c]Freelance Medical Doctor, Messina, Italy
[d]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina. Vill. SS. Annunziata 98168 Messina, Italy

Abstract

In the present study, we investigated the effects of *Kigelia africana* (Lam.) Benth. fruits ethanolic extract in prepubertal male rats, to evaluate the influence of the extract on the reproductive system and on pubertal development**.** Experiments were conducted using the rodent pubertal male assay. The plant extract, analyzed by TLC, HPLC-PDA and HPLC-ESI-MS, was administered orally at doses of 200, 400 and 800 mg/kg b.w. from post-natal date 21 to post-natal day 53. Age at puberty onset, body growth, development of sexual organs exposure to plant extract or positive control were examined. Results obtained indicate that *Kigelia* extract, at all doses tested, significantly anticipates puberty and increases body growth and sexual organs development. These effects appears to be due to stimulation of the secretion of androgenic hormones by the compounds found in its extract and scientifically support some of its traditional uses in disorders of the male reproductive system.

Keywords: *Kigelia africana*; Folk medicine; Phytochemical analysis; Immature rats; Pubertal assay; Reproductive system; Androgenic activity.

Experimental

Plant materials

Fresh mature fruits of *Kigelia africana* were collected in Limakole and Siby villages (Mali) in june 2016 (Micheli et al. 2016) and were authenticated at the Traditional Medicine Department (DMT), Faculty of Medicine, University of Bamako (Mali). The fruits have been air-dried, pulverized and stored in airtight containers until use. A voucher specimen (nr. 03128) was deposited in the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences of the University of Messina (Italy).

Preparation of extract

The extraction was done using the Soxhlet apparatus with ethanol as the solvent. A total of 50 g of the powdered fruit was used for the extraction and was packed into the thimble of the Soxhlet apparatus and a total of 625 mL of 60 % ethanol was used for the extraction. At the end of the extraction process, the extract was filtered and concentrated to dryness under vacuum in a rotatory

evaporator (Buchi R-205), at a temperature of 40°C and a pressure of 337 mbar. The yield which was 25.9% was stored in sterile universal containers and kept in the freezer for further use.

Phytochemical screening

TLC and HPLC-PDA, HPLC-ESI-MS *analysis*

For the determination of polyphenols and iridoids, 1 mg of dried ethanolic extract was dissolved in 1 mL of methanol. Aliquots of 30 μL each were applied onto the silica gel 60 F_{254nm} precoated plates (Merck, Germany), using ethyl acetate/formic acid/glacial acetic acid/water (100:11:11:27 v/v/v/v) as the mobile phase for polyphenols; and dichloromethane/methanol/water (80:20:2 v/v/v) for iridoids. The spots were located by plates exposure to UV light (λ = 365 nm). Polyphenols were detected with 2-aminoethyl diphenylborinate reagent (2-APB) and iridoids with anisaldehyde-sulfuric acid reagent (1:50) (ASA), both followed by heating at 110°C for 10 minutes. For the analysis of phytosterols, the ethanolic extract (2 g) was dissolved in H_2O (20 mL) and extracted at room temperature with benzene (3 x 20 mL, 30 min each) in a separatory funnel. Benzenic extracts were collected and evaporated to dryness, under reduced pressure and low temperature, in the rotary evaporator. Aliquots of 30 μL each were applied onto the silica gel 60 F_{254nm} precoated plates (Merck, Germany), using hexane/acetone (80:20 v/v) as the mobile phase. The plate was spectroscopically observed under UV light ((λ = 365 nm) and was developed with anisaldehyde-sulfuric acid reagent, followed by heating at 110°C for 10 min. Standard solutions with concentration of 0.1 mg/mL were prepared.

For a further standardization of phenolic and iridoid fractions of the ethanolic extract, high performance liquid chromatography with photodiode array (HPLC-PDA) and electrospray mass spectrometry (HPLC-ESI-MS) detection, were applied (Costa et al. 2017).

Animals and experimental design

Experimental procedures, according to protocols of the International Agencies (EDSTAC, EPA) with some modifications, were approved by animal welfare Committee University of Messina (ID 16/2016) and Ministry of Health (authorization number 814/2016 PR), Italy. In this investigation, 50 immature Sprague-Dawley male rats (21 days of age, 58-60 g b.w.) were used. The animals were obtained from the Laboratory of Envigo RMS of Udine, Italy. They were kept under standard laboratory conditions (12 h light: 12 h dark and at 25 ± 2 °C). They were allowed unrestricted access to water and commercial rat pellets (Envigo RMS Srl, Udine, Italy). Animals were randomly divided into 5 groups (A, B, C, D and E) of 10 animals per group. Group A (negative control) animals received only vehicle, normal saline/alcohol; Group B (positive control) received 1 mg/kg of testosterone propionate (Sigma Chemical Co., St. Louis, MO), while groups C, D and E received 200, 400 and 800 mg/kg of *K. africana* fruit extract respectively daily for duration of treatment. All

groups were treated from post-natal date 21 to post-natal date day 53 by oral gavage (plant extract), except testosterone group that was treated for subcutaneous route. During the treatment period, daily clinical observation was performed on behavior of all animals to detect any signs of toxicity effects like dyspnea or shortness breath, seizures, restiveness or dizziness etc. (by Irwin test) or preputial separation (onset of puberty). In addition, all animals were weighed and measured (snout–tail length) weekly and at the time of puberty onset. At the end of the experimental period, changes in body weight and body length, ~~age at puberty onset (preputial separation)~~, endpoints associated with the development of the reproductive system were examined (Clark 1999). Rats were sacrificed on the 53^{th} day by anesthesia with sodium pentobarbital (60 mg/kg body weight), by cervical dislocation and the ~~sex organ, secondary sexual characteristics including~~ reproductive organs (testis, seminal vesicles, epididymis, vas deferens and prostate glands) weights and macroscopic morphology of organs exposure to plant extract or positive and negative control compound were examined.

Statistical analysis

Results are expressed as mean ± standard deviation and subjected to statistical analysis using analysis of variance (ANOVA) followed by Dunnett's test. The significant level considered was $P < 0.05$.

Table 1S: Thin layer chromatography data of polyphenols, phytosterols and iridoids in ethanolic and benzenic extracts of *K. africana*

	Rf (10 cm)	**Ethanolic extract**			**Benzenic extract**	
		365 nm	2-APB	ASA	365 nm	ASA
Polyphenols						
Rutin	0.4	+	Yellow			
Caffeic acid	0.9	+	Light-blue			
Ferulic acid	0.9-0.95	+	Light-blue			
Chlorogenic acid	0.45	+	Green-light-blue			
p-Coumaric acid	0.5	+	ND			
Phytosterols						
β-Sitosterol	0.5				+	Violet
Stigmasterol	0.5				+	Violet
Iridoids						
Minecoside	5.8	+		Dark-Orange		

2-APB: 2-aminoethyl diphenylborinate; ASA: anisaldehyde-sulfuric acid; ND: not detected

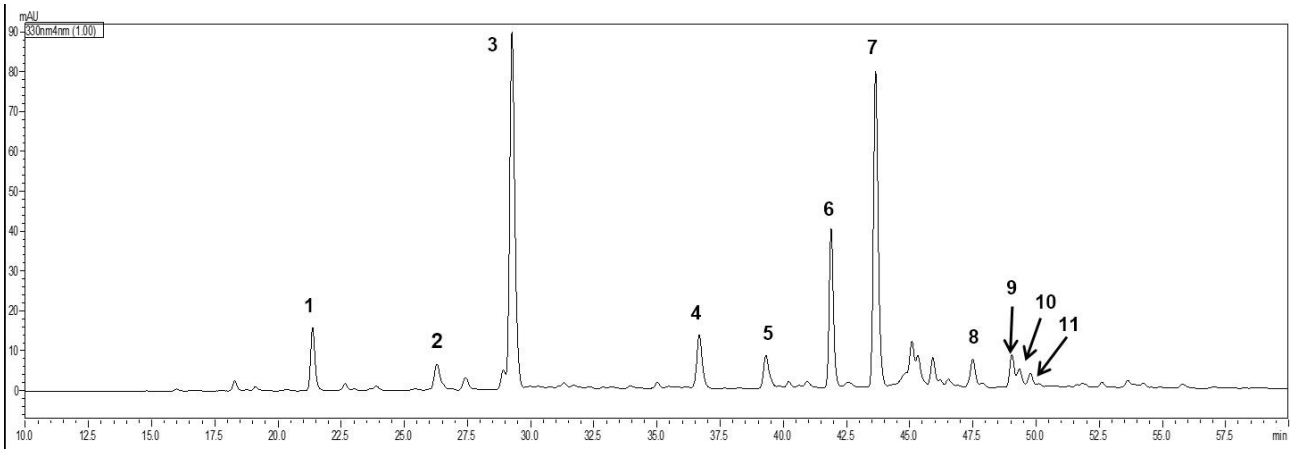

Figure 1S – HPLC-PDA chromatogram of ethanolic extract of *Kigelia africana*. Peak#1: caffeic acid glucoside; peak#2: p-coumaroyl glucose; peak#3: caffeic acid; peak#4: p-coumaric acid; peak#5: ferulic acid; peak#6: verbascoside; peak#7: verminoside; peak#8: unkown 1; peak#9: unknown 2; peak#10: specioside; peak#11: minecoside.

Table 2S: Effects of *K. africana* ethanolic fruit extract on Pubertal Development and on body growth of immature male rats.

Treatment	Dose	Puberty onset		Weight	Length
	mg/Kg	Animal (%)	Age (days)	(% increase)	(% increase)
Control	_	100	47±1.5	162	46
Testosteron	1	100	39±0*	175*	59*
Extract	200	100	42±1.0*	175*	57*
“	400	100	41±0*	180*	58*
“	800	100	41.5±0.5*	179*	58*

*Values are expressed as Means ± S.D; N=10. *P<0.05*

Table 3S: Effects of *K. africana* ethanolic extract on sexual organs development of male rats.

Treatment	**Dose** mg/Kg	**Testes (g)**	**Epididymis** (mg)	**Seminal vesicles** (mg)	**Prostate** (mg)	**Vas-deferens** (mg)
Control	_	2.9±0.3	280±2.5	530±0.3	200±0.4	85±4.0
Testosteron	1	3.5±0.4**	298±1.8*	790±0.1**	440±0.1**	108±3.5*
Extract	200	3.4±0.2**	294±3.0*	840±0.5**	265±0.5**	97±2.9*
“	400	3.7±0.03**	297±2.7*	850±0.1**	270±0.3**	101±4.5*
“	800	3.6±0.05**	296±2.0*	855±0.3**	278±0.6**	104±5.0*

*Values are expressed as Means ± S.D; N=10. *P<0.05, ** P<0.01*