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·*Complementary Medicine*·

Effects of *Basella alba* and *Hibiscus macranthus* extracts on testosterone production of adult rat and bull Leydig cells

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Abstract

Aim: To determine the androgenic effects of *Basella alba* and *Hibiscus macranthus* extracts in the rat and the bull, and to develop a novel *in vitro* test system using Leydig cells from bull testes. **Methods:** The effect of methanol extracts from both plants on testosterone production in isolated Leydig cells from the rat and the bull was analyzed using ^{125}I-radioimmunoassay (^{125}I-RIA). Rat Leydig cells were obtained by common methods, whereas a novel technique was used to purify Leydig cells from bull testes. **Results:** Bull testes from the slaughter house were a cheap source of pure Leydig cells. In culture, these cells produced testosterone for 5-6 days, which can be stimulated by human chorionic gonadotrophin (hCG). *Basella alba* extracts significantly enhanced testosterone production in bull and rat Leydig cells in a concentration-dependent manner. *Hibiscus macranthus* showed no androgenic effect but was shown to inhibit testosterone production at higher concentrations. **Conclusion:** Leydig cells purified from bull testes can be used as an alternative tool in experimental animal research. Certain fractions of *Basella alba* extract demonstrated androgenic potential whereas *Hibiscus macranthus* extracts did not. *(Asian J Androl 2005 Dec; 7: 411–417)*

Keywords: bull; rats; Leydig cells; testosterone; *Basella alba*; *Hibiscus macranthus*; plant extract

1 Introduction

Hibiscus macranthus, *Basella alba* and many other indigenous plants from Cameroon have been shown to have an effect on male reproductive function [1–3]. Indeed, the aqueous extract obtained from the mixture of the leaves of both plants is used in the Western Province of Cameroon to cure male sexual asthenia and infertility. Previous works on the aqueous extract demonstrated their androgenic effect [1, 4]. To better understand the exact potential of these plants in reproductive health, we analyzed their mechanism of action in more detail using extracts of different organic solvents. However, previous works using rat testis slices failed to reveal the active principles or fractions (Beboy, unpublished data, 2001). Thus it is necessary to obtain a new *in vitro* test system to analyze the androgenic effect of

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the active plant fractions. For this purpose, a novel method for the purification of Leydig cells from bull testes was developed. Bull testes are a by-product of the local slaughter house and are used in this paper as an alternative material in laboratory research. These Leydig cells as well as those obtained from rats by common purification methods were used to test the effect of the methanol extracts from both plants and the higher purified fractions from *Basella alba* on their testosterone production.

2 Materials and methods

2.1 Chemicals

Dubecco's Modified Eagle's medium-F12 Ham nutrient mixture (DMEM/Ham F12) (Gibco, Berlin, Germany), Collagenase Type I, Soybean Trypsin inhibitor (STI), DNAse, insulin, transferrin, vitamin E, penicillin and streptomycin were all obtained from Sigma (Deisenhofen, Germany).

2.2 Preparation of the extracts and fractions

Air-dried leaves of both *Hibiscus macranthus* (0.5 kg) and *Basella alba* (0.5 kg) , obtained from Batoufam (Western Province, Cameroon) were powdered in a mill. Each powder was then subjected to an organic solvent extraction by maceration under gentle agitation in a glass vessel for 48 h at room temperature using successively hexane (500 mL for 5 h, three times), methylene chloride (500 mL for 5 h, three times) and methanol (500 mL for 6 h, three times). The methanol extracts of both *Hibiscus macranthus* and *Basella alba* were concentrated under vacuum for dryness. The greenish viscous residues (12 g for *Hibiscus macranthus* and 15 g for *Basella alba*) were obtained and tested. The extract from *Basella alba* was further subjected to flash chromatography (60 mm × 55 mm column size) on silica gel (230–400 mesh) as a stationary phase. The extract was added in the form of dry powder to the column. Elution was done using a CH_2Cl_2/MeOH system with an increasing polarity (100 % to 50 % CH_2Cl_2) as the mobile phase. Eighty-one fractions of 100 mL each were collected and evaporated under vacuum at 50 °C and pooled according to their retention factor (Rf) on thin layer chromatography (TLC) using a CH_2Cl_2/MeOH system as eluent. Twelve main fractions labeled A to L were obtained. The above fractions were eluted with the following CH_2Cl_2/MeOH system polarities: A (100/0), B and C (95/5), D and E (90/10), F and G (85/15), H (75/25), I, J and K (50/50) and L (0/100). All these fractions were further analyzed for the presence of certain classes of phytochemical compounds using usual organic phytochemical screening methods [5] (see Table 1 for details). The hexane and methylene chloride extracts were not used for the lack of androgenic activities (Beboy, unpublished data, 2001).

2.3 Purification and culture of Leydig cells from bull and rat

Adult testes were collected in the local slaughter house (Giessen, Germany) and were used within 1 h after isolation. Testes were washed with tap water, blotted and cleaned with ethanol (70 %). Connective tissues was removed by dissection and the testis was aseptically introduced into a sterile hood. The tunicia albuginea was then removed by dissection using scalpels and scissors, then a piece of the testis was cut and weighed (7 g–10 g), washed twice with DMEM/Ham F12 medium containing gentamicin and cut into thin slices (0.5 mm^2). Then it was washed again with the DMEM/Ham F12 medium and quantitatively transferred into 20 mL of dissociation medium (DMEM/Ham F12 containing 2 mg/mL collagenase type I, 0.2 mg/mL STI and 20 µg/mL DNAse) and incubated for 20 min at 34 °C under constant shaking. Thereafter another 20 mL of DMEM/Ham F12 medium was added and the digested mixture was allowed to settle. The supernatant was carefully collected using a pipette and put into a sterile tube. The remaining undigested tissue was subjected to two repeating digestion steps as described above. All the supernatants were pooled and centrifuged at 180 × *g* for 10 min at room temperature. The pellet, containing the interstitial cells, was washed three times with DMEM/Ham F12 medium and filtered through a 70 µm nylon mesh (Falcon, Becton Dickinson, NJ, USA).

Bull Leydig cells were further purified using two different discontinuous percoll gradients by a modification of the method described by Brun *et al.* [6]. The first gradient consisted of 5 %, 30 %, 58 % and 70 % percoll, four phases. The filtered cell suspension was placed over the gradient and centrifuged at 1100 ×*g* for 20 min at 18 °C. The second band (cells situated between the 30 % and the 58 % percoll phases) was collected, washed with DMEM/Ham F12 medium, and layered on a second gradient (30 % to 60 % percoll). The gradient was centrifuged as above. Three bands found at 40 %, 45 % and 50 % were collected, mixed and washed with DMEM/Ham F12 medium. The cells were counted using a hemocytometer.

They were characterized by 3β-hydroxy- steroid dehydrogenase (3β-HSD) histochemistry [7] and hCG stimulated testosterone production (for 12 h). The purity of cells was about 92 % and viability >95 % as detected by trypan blue exclusion.

Leydig cells were also obtained from adult Sprague–Dawley rats (90 days old) after three enzymatic dissociations of the testis using collagenase (0.5 mg/mL), Dnase (20 μg/mL) and STI (0.05 mg/mL) in DMEM/Ham F12 at 34 ºC for 10 min. They were further purified on a percoll gradient by the method described by Papadopoulos *et al*.[8].

Leydig cells were used for experiments just after purification and cultivated in DMEM/Ham F12 medium containing 10 μg/mL insulin, 5 μg/mL transferrin and 10 μg/mL vitamin E, 100 U/mL penicillin and 100 pg/mL streptomycin in 96 well plates (50×10^3 cells in 0.2 mL).

2.4 Effects of extracts and fractions on Leydig cells

Methanol extract was dissolved in Dimethylsulfoxide (DMSO) and added to cells in culture (0.01 μg–100.00 μg extract/mL final concentration in culture medium). The control well contained DMSO to a level not exceeding 0.5 %. After 12 h, medium was collected from each well and testosterone assayed by ^{125}I-radioimmunoassay (^{125}I-RIA) (DSL, Sinsheim, Germany) (detection limit: 0.006 ng/mL, Intra assay variation 5 %). Testosterone production by fractions obtained from *Basella alba* methanol extract were additionally tested after 4 h of incubation time.

Extracts mixed to culture medium without Leydig cells were also assayed for the presence of testosterone. The RIA count obtained were even higher than the zero concentration of the standard. This means the compounds present in the extract do not cross react with the testosterone assay system.

2.5 Statistical analysis

All data obtained were analyzed using MedCalc computer program (MedCal Software, Mariakerke, Belgium). They were analyzed for normal distribution using the Kolmogorov–Smirnow test, before any further analysis either by one-way ANOVA or Kruskal–Wallis test. When ANOVA was significant, *t*-test was used for pair comparison between means.

3 Results

3.1 Bull Leydig cells: purity and testosterone production

From 11 different purifications, the number of Leydig cells collected was $(2.4 \pm 1.0) \times 10^6$/g of testis, with a viability of 95 %–98 % (Figure 1). Testosterone production in these cells was significantly stimulated by human chorionic gonadotrophin (Figure 2). When the cells were cultured without changing the medium, testosterone was produced continuously reaching a plateau after 5 days (Figure 3). When the medium was changed every day, we obtained a rapid decline of testosterone production within 2–3 days (data not shown).

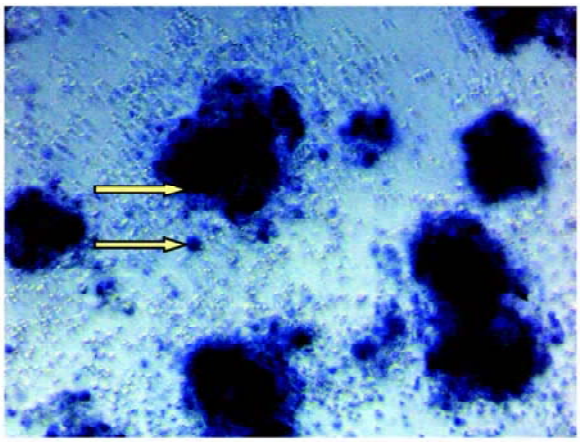

Figure 1. 3β-HSD histochemical staining of freshly purified Leydig cells from bull testis. Cell cluster (upper arrow) and isolated cells (lower arrow) were shown.

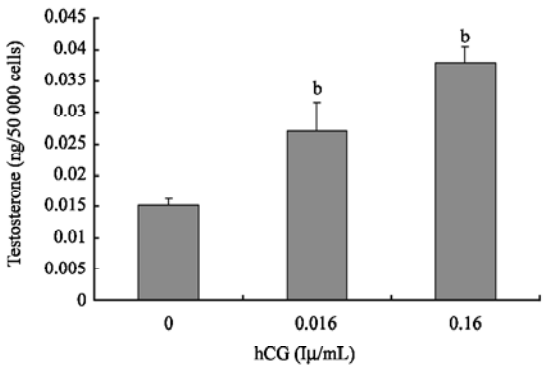

Figure 2. Testosterone level in cultured Leydig cells from a bull after 12 h under hCG stimulation. Cells were cultured in DMEM/Ham F12 medium containing insulin 10 μg/mL, transferrin 5 μg/mL, vitamin E 10 μg/mL, penicillin 100 U/mL and streptomycin 100 μg/mL. $^bP < 0.05$, compared with the control.

3.2 Effect of Basella alba or Hibiscus macranthus methanol extract on testosterone production in rat Leydig cells

Methanol extracts of *Basella alba* caused a significant ($P < 0.001$, ANOVA) concentration-dependent increase in testosterone production after 12 h exposure to rat Leydig cells. We observed 190 % and 55 % increased testosterone levels, respectively, compared with the control and hCG-stimulated control containing DMSO for a concentration of 10 µg/mL *Basella alba* extract (Table 1). When DMSO was not added in the hCG control, a 2.5-fold stimulation of testosterone production was observed. In contrast, extracts from *Hibiscus macranthus* increased the level of testosterone slightly at 10 µg/mL, but decreased testosterone production significantly by 60 % compared with the control at 100 µg/mL ($P < 0.05$, paired *t*-test).

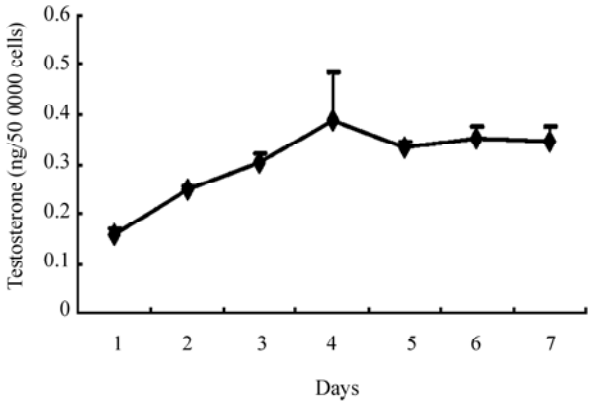

Figure 3. Effect of 1-week culture without medium renewal on testosterone production by Leydig cells from bull testis. The cells were cultured in DMEM/Ham F12 medium containing insulin 10 µg/mL, transferrin 5 µg/mL, vitamin E 10 µg/mL, penicillin 100 U/mL and streptomycin 100 µg/mL. Values were mean ± SD from four different incubations with three replications.

3.3 Effect of different fractions from Basella alba on testosterone production of Leydig cells from bull

The methanol extract of *Basella alba* was further separated by silica gel column chromatography. When bull Leydig cells were incubated with various fractions (100 µg/mL each) for 12 h, significantly ($P < 0.05$, paired *t*-test) enhanced testosterone levels compared with the control were obtained in culture supernatants containing fractions B, J", K, K' and L and hCG control not containing DMSO (Figure 4). When bull Leydig cells were incubated with different concentrations (10 µg/mL and 100 µg/mL) of these fractions for 4 h, testosterone was only detectable in incubations containing hCG without DMSO and fraction B (Table 2).

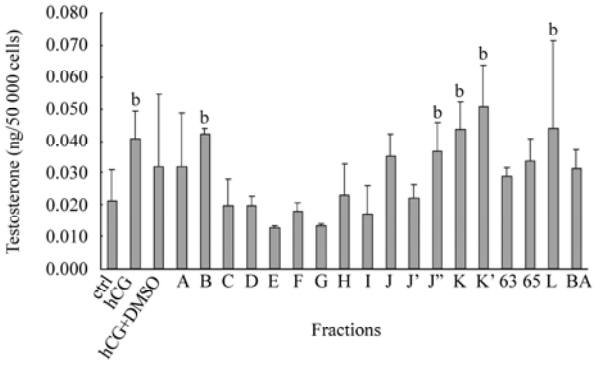

Figure 4. Testosterone level in cultured Leydig cells from bull after 12 h in the presence of various fractions obtained from *Basella alba* methanol extracts. Control contained 0.02 % DMSO or hCG (0.016 IU/mL). The fractions (in alphabetical order) were diluted in DMSO and added at a final concentration of 100 µg/mL to the cells in DMEM/F-12 medium containing insulin 10 µg/mL, transferrin 5 µg/mL, vitamin E 10 µg/mL, penicillin 100 U/mL and streptomycin 100 µg/mL. Fractions 63 and 65 are cristals obtained during collection of fractions. Values were mean ± SD from four different incubations of 12 h. $^bP < 0.05$, compared with the control.

Table 1. Testosterone levels in 12 h cultured Leydig cells from rats in the presence of various concentrations (µg/mL) of *Basella alba* and *Hibiscus macranthus* methanol extracts. Values were mean ± SD from four different incubations of 12 h. The control incubations contained DMSO (<0.5 %) or hCG at the level of 0.016 IU/mL. The extracts were diluted in DMSO and added at final concentrations of (0.01–100)µg/mL to the cells in culture in DMEM/Ham F12 medium containing insulin 10 µg/mL, transferrin 5 µg/mL, vitamin E 10 µg/mL, penicillin 100 U/mL and streptomycin 100 µg/mL. $^bP < 0.05$, compared with control and the hCG-stimulated control. DMSO, dimethylsulfoxide; hCG, human chorionic gonadotrophin.

Concentrations		Testosterone level (ng/50 000 cells)						
extracts	Control	hCG	hCG + DMSO	0.01	0.1	1	10	100
Basella alba	0.050 ± 0.003	0.176 ± 0.046^b	0.094 ± 0.007^b	0.050 ± 0.040	0.114 ± 0.008^b	0.139 ± 0.028^b	0.145 ± 0.007^b	0.125 ± 0.037^b
Hibiscus macranthus				0.063 ± 0.009	0.062 ± 0.015	0.062 ± 0.010	0.082 ± 0.018	0.019 ± 0.016^b

3.4 Testosterone production of rat Leydig cells in presence of various concentrations of Basella alba fraction B

Fraction B seems to contain most of the active principle of *Basella alba*. Therefore, we also analyzed the effect of various concentrations of Fraction B on rat Leydig cell culture. After 4 h of stimulation, a concentration-dependent increase (r = 0.89) of testosterone production was observed (Table 3). Significant higher hormone levels compared with the control were achieved after stimulation with 10 μg/mL or higher of fraction B (P = 0.02, Kruskal-Wallis test).

3.5 Phytochemical composition of various fractions

The results of the phytochemical test were shown on Table 4. Terpenoids and courmarins were the major classes of compounds found in both *Hibiscus macranthus* and *Basella alba* fractions. Some limonoids were also present in *Basella alba.* On TLC plates of the active fraction B two spots with Rf values of 0.43 and 0.57, respectively, were observed (data not shown; elution solvent:

Table 2. Testosterone levels in 4 h cultured Leydig cells from bull in the presence of various concentrations (μg/mL) of *Basella alba* fractions B, J'', K and L. Values were mean ± SD from four different incubations of 4 h. The control incubations contained DMSO (< 0.5 %) or hCG (0.016 IU/mL). The extracts were diluted in DMSO and added at a final concentration of (0.01–100) μg/mL to the cells in culture in DMEM/Ham F12 medium containing insulin 10 μg/mL, transferrin 5 μg/mL, vitamin E 10 μg/mL, penicillin 100 U/mL and streptomycin 100 μg/mL. [b]P < 0.05, compared with hCG-stimulated control. DMSO, dimethylsulfoxide; hCG, human chorionic gonadotrophin; ND, not detectable.

	Control	hCG	hCG + DMSO	B (μg/mL)		J'' (μg/mL)		K (μg/mL)		L (μg/mL)	
				10	100	10	100	10	100	10	100
Testosterone level (ng/50 000 cells)	ND	0.016 ± 0.007	ND	0.033 ± 0.004	0.120 ± 0.004[b]	ND	ND	ND	ND	ND	ND

Table 3. Testosterone level in 4 h cultured Leydig cells from rat in the presence of various concentrations (μg/mL) of *Basella alba* fraction B. Values were mean ± SD from four different incubations of 4 h. The control incubations contained DMSO (<0.5%) or hCG (0.016 IU/mL). The fraction was diluted in DMSO and added to the cells in culture in DMEM/Ham F12 medium containing insulin 10 μg/mL, transferrin 5 μg/mL, vitamin E 10 μg/mL, penicillin 100 U/mL and streptomycin 100 μg/mL. [b]P<0.05 (Kruskal – Wallis test), compared with control. Spearman rank correlation r = 0.89, P = 0.0004; hCG, human chorionic gonadotrophin.

	Control	hCG	*Basella alba* fractions B (μg/mL)				
			0.1	1	10	100	500
Testosterone level (ng/50 000 cells)	0.016 ± 0.004	0.054 ± 0.042	0.024 ± 0.011	0.022 ± 0.011	0.033 ± 0.009[b]	0.171 ± 0.011[b]	0.419 ± 0.084[b]

Table 4. Results of phytochemical tests on the different fractions obtained from *Basella alba* or *Hibiscus macranthus* methanol extracts. (+), presence of compounds; (–), absence of compounds; (/), not tested. The different groups of compounds were dectected by their specific corolarations in the presence of the following reagents [5]: [a]solid Mg in ethyl alcohol and drops of hydrochloric acid acid; [b]ammonia vapor; [c]Libermann–Burchard test using acetic anhydride sulfuric acid and chloroform; [d]Molish test for sugars using α-naphtol; [e]Erlich test using 4-(N,N-dimethyamino) benzaldehyde.

Species	*Basella alba* Fractions												*Hibiscus macranthus* Fractions							
Class of compounds	A	B	C	D	E	F	G	H	I	J	K	L	F_A	F_B	F_C	F_D	F_E	F_F	F_G	F_H
Flavonoids[a]	–	–	–	–	–	–	–	+	–	–	–	–	/	/	/	/	/	/	/	/
Coumarins[b]	–	–	–	–	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	+
Terpenoids or sterols[c]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–
Sugars[d]	–	–	–	–	–	–	–	–	+	+	+	+	/	/	/	/	/	/	/	/
Limonoides[e]	–	–	+	+	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–

methylene chloride: methanol [97:3 v/v]).

4 Discussion

In vitro cell culture is a useful tool to analyze various functions of Leydig cells. This technique was used to explore the direct effects of hormones, paracrine factors or xenobiotics on Leydig cells [6, 9–14]. However, these cell preparations were of various purity according to the type of biochemical study investigated. In most cases Leydig cells were purified from laboratory animals such as the rat and mouse. This fact is often criticized by animal protection associations. The purification of Leydig cells from bull testes – waste products from the slaughter house – is a contribution to the development of an alternative animal test method in laboratory research.

Purification of Leydig cells from bulls have not yet been described in the scientific literatures. But it is known that Leydig cells are colored in blue by 3β-HSD histochemistry and upon centrifugation on a percoll gradient, they are usually found between gradient concentrations 35 % and 60 %, which corresponds to buoyant densities of 1.067–1.075 [15]. All these characteristics were exploited advantageously in a procedure described herein which combines two percoll gradients. The cells purified from the bull testes produced testosterone and were stimulated by hCG. When they were cultured with the medium changed every day, they had a rapid decline of testosterone production. This may be because of our culture conditions were not optimal for bull Leydig cells. Changing the medium may have affected them more rapidly. However, when the cells were cultured without changing the medium, testosterone was produced continuously reaching a plateau after 5–6 days. This could mean that in our culture conditions, these Leydig cells are functional up till day 5. Our purified Leydig cells were used in the following experiments without changing the medium. When methanol extracts were added to rat Leydig cell cultures, it appeared clearly that, *Basella alba* was the plant responsible for the induction of testosterone production, since the production of this hormone in the medium increased with increasing concentrations of its extract. In contrast, *Hibiscus macranthus* methanol extract had no androgenic effect but inhibited testosterone production at a concentration of 100 µg/mL. The leaves of this plant are also used in a different herbal medication together with other three different plants, for fertility regulation in women. It has been recently shown that this herbal medication has estrogenic properties [16, 17].

Based on the above results, only fractions from *Basella alba* were further analyzed for their androgenic activities. In purified bull Leydig cells, many of the fractions tested (mainly B, J", K, K' and L) induced testosterone production after 12 h exposition. Usually, for studies on the effects of various compounds on Leydig cell function, these cells are incubated for 3–5 h [6, 14, 18, 19]. Thus, Leydig cells were also incubated for 4 h with the above named fractions in order to compare the response to that obtained after a 12-hour incubation period. Here, testosterone was found only in incubations stimulated by fraction B. In other incubations, including the controls, testosterone levels were below the detection limit of the RIA (0.006 ng/mL). Generally, the levels of testosterone produced by the purified Leydig cells from bull in this study was lower compared with those of rats. That might be the reason why testosterone was not detected in the control. Moreover, fraction B of *Basella alba* caused a concentration-dependent increase in testosterone production in rats Leydig cells culture, thus confirming the results obtained with bull Leydig cells. This suggested fraction B as the active one. As shown by TLC analysis, fraction B is a mixture of two fluorescent products with Rf values of 0.43 and 0.57 (on CH_2Cl_2/methanol 97: 3 v/v solvent system), respectively. Phytochemical tests demonstrated the presence of terpenoid or steroid compounds in these fractions. Some steroid compounds such as cholesterol or dehydroepiandrosterone are known as a precursor of the biosynthesis of testosterone. Further studies will probably help to elucidate the structure of compounds responsible for androgenic activity.

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