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·*Complementary Medicine*·

Effects of extracts from *Hibiscus macranthus* and *Basella alba* mixture on testosterone production *in vitro* in adult rat testes slices

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Abstract

Aim: To find an *in vitro* system for the measurement of the androgenic effects of different extracts of *Hibiscus macranthus* (Malvaceae) and *Basella alba* (Basellaceae). **Methods:** The production of testosterone from testes slices incubated in two media, either Krebs-Henseleit buffer containing 0.5 % Bovine serum albumin (BSA) or Dubecco's Modified Eagle's medium-F12 Ham nutrient mixture (DME/Ham F12), under a mixture of 5 % CO_2 in 95 % air was determined either in the presence or absence of cofactors and *Hibiscus macranthus* plus *Basella alba* (HMBA) extracts. **Results:** The testosterone production was increased in testes slices incubated in DME/Ham F12 medium in response to the cofactors (49 %) and aqueous extracts (34 %–60 % according to dilutions). Under the same atmospheric conditions, there was no positive response of the testes slices to either cofactor or HMBA extract stimulation in Krebs-Henseleit buffer containing 0.5 % BSA. In further investigations related to the effect of HMBA, the DME/Ham F12 medium was used. The results obtained from the *in vitro* test showed that the activity was present mainly in methylene chloride and methanol, since these extracts induced an increase in testosterone production by testes slices. **Conclusion:** The testes slice system is suitable to be used for further *in vitro* investigations of the isolation of androgenic bioactive components of plants. *(Asian J Androl 2006 Jan; 8: 111–114)*

Keywords: testis; testosterone; plant extracts; *Hibiscus macranthus*; *Basella alba*

1 Introduction

Hibiscus macranthus, *Basella alba* and many other indigenous plants have been shown to improve male reproductive function [1–4]. In the Western province of Cameroon, traditional healers use the mixture of the two plants to prepare a crude extract which improves male virility. We previously reported that the aqueous extract obtained from a mixture of fresh or dried leaves of the two plants increased testosterone production in adult male rats. The testes of rats in the extract showed abundant spermatozoa in the lumen of seminiferous tubulus and a high production of testosterone was observed from the testes slices [1]. This implied that the extract possesses

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active components that increase steroidogenesis. In continution of previous work, the effect of different extracts from a mixture of the two plants were studied *in vitro* using adult rat testes slices in the present study. The purpose of this study was to determine a system which would allow the testing of different fractions obtained during isolation of bioactive components and to find a better solvent system for obtaining active extracts.

2 Materials and methods

2.1 Chemicals

NAD+, NADP+, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (from torula yeast), bovine serum albumine (BSA), Dubecco's Modified Eagle's medium-F12 Ham nutrient mixture 1:1 (DME/Ham F12), penicillin and streptomycin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Testosterone assay kit was purchased from World Health Organisation (WHO) (Immunometric, London, UK) and ICN Biochemicals Inc. (Carson, CA, USA). Other reagents used for Krebs-Henseleit buffer preparation or for steroid extraction were of the highest purity grade.

2.2 Plant extracts

Dry leaves of *Hibiscus macranthus* Hochst ex A. Rich (HM) and *Basella alba* L. (BA) collected in Batoufam (western province of Cameroon) in June 1996 were grounded using an electric grinder. Botanical identification was done by Dr Onana and specimens deposited in the National Herbarium (IRAD Yaoundé, Cameroon), vouchers No. 41 881 and No. 40 720, respectively.

The aqueous extracts were obtained by mixing 18 g dry leaves of HM and 9 g dry leaves of BA in distilled water (1 L) with constant stirring for 12 h at least. The mixture was then filtered and the filtrate stored at –20 °C.

A powdered mixture (100 g) of leaves of the two plants (HM and BA 2:1 [w/w]) was successively extracted by maceration with hexane, methylene chloride and methanol. After evaporation to dryness under vacuum at 40 °C, the respective yields obtained were 3.1 %, 1.5 % and 6.7 %. All the extracts were concentrated and kept at 4 °C.

2.3 In vitro production of testosterone by testes slices

Testes were removed by dissection from male adult albino rats (Wistar strain) aged 90 days and killed by decapitation. After removal of the connectives tissues, slices were cut (20 mg ± 2 mg) and put in culture tubes containing 0.5 mL of either DME/Ham F12 medium (containing 1.2 g/L sodium bicarbonate) or Krebs-Henseleit buffer [5] which supplemented with 1 g/L glucose, 5 mg/mL BSA and antibiotics (50 IU/mL penicillin and 50 µg/mL streptomycin). Testes slices obtained from the same animal were used for each set of incubation. During incubation, a positive control containing the enzymatic cofactors solution (0.4 mmol/L NAD+, 0.4 mmol/L NADP+, 3.5 mmol/L glucose 6-phosphate and 2 IU/mL glucose 6-phosphate dehydrogenase) [6] was run in parallel with a control without the plant extract. The aqueous extract previously tested in the *in vivo* experiments was analysed in the two culture media (25 µL) at different dilutions. The incubations were conducted at 34 °C under CO_2/air 5:95 (v/v) for 2 h. All the organic extracts dissolved in dimethysulfoxide (DMSO) were tested (25 µL) for their ability to induce secretion of testosterone by the testes slices incubated in DME/Ham F12 medium under the same conditions as described above. The control contained DMSO (< 1 %).

2.4 Testosterone assay

After incubation, each tube was homogenized using a Tenbreok homogenizer (Fischer Bioblock Scientific, Illkirch, France) and centrifuged at 4 °C (1 200 × *g* for 10 min). Testosterone was extracted from the supernatant using ethyl ether/chloroform mixture (3:1 [v/v]) and assayed according to WHO method [7] by ^{3}H-RIA using mouse monoclonal antibody, or by ^{125}I-RIA in ICN protocols. In both cases the cross reaction of the antibody with other androgens was less than 1.8 %. The intra assay variation was 5 %.

2.5 Statistical analysis

Data were expressed as mean ± SD. One- and two-way ANOVA were used for statistical comparison of the data. Differences between groups were assessed by paired *t*-test [8].

3 Results

3.1 Testosterone production under 5 % CO_2 in air condition

Under controlled air conditions, the testosterone production was similar for the control in both media (Table 1), but the effects of enzymatic cofactors (positive control) and aqueous plant extract were increased significantly in

Table 1. Testosterone production in testes slices after incubation in either Krebs-Henseleit buffer containing 0.5 % BSA or DME/Ham F12 under 5 % CO_2/95 % air condition in the presence of various dilutions of HM-BA (aqueous extract from a mixture of *Hibiscus macranthus* and *Basella alba* leaves). Testosterone was assayed using ^{3}H-RIA kit from the WHO. Mean ± SD, $^bP < 0.05$, $^cP < 0.01$ (paired *t*-test), $^fP < 0.01$ (ANOVA), compared with the corresponding control.

	Testosterone (ng/mg)	
	Krebs-Henseleit (0.5 % BSA)	DME/Ham F12
Control	0.076 ± 0.025	0.077 ± 0.011
Positive control	0.091 ± 0.008	0.116 ± 0.014^f
HM-BA dilution 1/500	0.040 ± 0.002	0.078 ± 0.008
HM-BA dilution 1/250	0.076 ± 0.024	0.104 ± 0.022^b
HM-BA dilution 1/50	0.094 ± 0.007	0.124 ± 0.008^c
HM-BA dilution 1/10	0.088 ± 0.017	0.092 ± 0.024

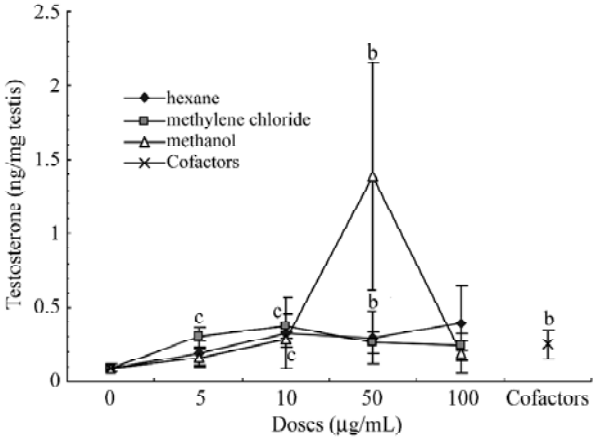

Figure 1. Testosterone level (ng/mg) in testes slices incubations in the presence of hexane, methylene chloride, methanol extracts from dry leaves of *Hibiscus macranthus* (HM) and *Basella alba* (BA), the control (0 μg/mL) and cofactors solution. Values are mean ± SD of 3 incubations. The methylene chloride extract (5 μg/mL, 10 μg/mL and 50 μg/mL) and the methanol extract (10 μg/mL and 50 μg/mL) induced a significant increase of testosterone production *in vitro*. $^bP < 0.05$, $^cP < 0.01$ (paired *t*-test), compared with the control. Testosterone was assayed using ^{125}I-RIA kit from ICN.

DME/Ham F12 medium when compared to the control ($P < 0.01$). The testosterone production percentages were calculated using the following formula: (Ti-Tc)/Tc × 100. T: for testosterone value, i: for positive control or extracts and c: for control. The testosterone production was 49 % higher in the positive control, and 34 % (1/250 dilution)–60 % (1/50 dilution) higher in incubations containing the aqueous extract when compared to the control in DME/Ham F12 medium. In Krebs buffer, the effects of both enzymatic cofactor solution (positive control) and plant extracts did not show significant differences when compared with the control.

3.2 Effect of extracts on testosterone production in vitro

The data shown in Figure 1 summarized the testosterone production under the influence of the different extracts. The methylene chloride extract (5 μg/mL, 10 μg/mL and 50 μg/mL) obtained from powdered leaves induced a significant increase of testosterone production *in vitro*. The methanol extract (10 μg/mL and 50 μg/mL) was also active in inducing testosterone production; although testosterone levels were high in incubations containing the hexane extract, they were not statistically significant compared with the control.

4 Discussion

The testes slices were used because we did not have the means of isolating Leydig cells, however, this was advantageous because our assays have been done with intact cells in their environment. Indeed it has been shown that testosterone production by Leydig cells is improved in the presence of factors from Sertoli and germ cell origins [9–13].

Under 5 % CO_2 in air conditions, the effects of the enzymatic cofactors as well as those of the plant extracts were more pronounced and reproducible on testes slices incubated in serum-free DME/Ham F12 medium (Table 1). The induction of testosterone production by the cofactors solution could be explained by the direct action of the Nicotinamide adenine dinucleotide phosphate-reduced form (NADPH) produced by the enzymatic cofactor solution in the process of testosterone synthesis since the enzymes involved are NADPH-dependent [14]. With increasing concentrations of the aqueous extract, we observed an enhancement of the testosterone production in DME/Ham F12 medium by rat testes slices; however a plateau was reached with a 1/50 dilution.

These experiments revealed that Krebs-Henseleit buffer containing 0.5 % BSA is a poor medium, since it failed to give reproducible results either for the positive control or for the plant extracts, unlike the serum-free

medium, DME/Ham F12. For this reason all further experiments with plant extracts were conducted in DME/Ham F12.

A mixture of the organic solvent extractions of both plants was tested because this is the form in which the plants are used by the tradipractitioner. Use of these extracts produced higher levels of testosterone in the incubation medium containing methylene chloride or methanol extracts. This means that these solvents have the ability to solubilize bioactive androgenic components of the plants. The stimulation of testosterone production was done in a dose-dependant manner as observed with the aqueous extract, however, a decrease was observed with 100 µg/mL mathanol extract.

We have shown in this study a direct action of the aqueous plant extracts on testes slices incubated in DME/Ham F12 medium under CO_2/air 5/95 (v/v), and further demonstrated that the activity of the mixture of the two plants is conserved in their organic extracts, mainly methylene chloride and methanol, obtained from the powder of the two plants. This *in vitro* test system as well as the procedure used for extraction will be useful for studies concerning the identification of bioactive components from plant extracts in the future.

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