REPRODUCTIVE BIOLOGY

Effect of different fractions from hydroalcoholic extract of Black Maca (*Lepidium meyenii*) on testicular function in adult male rats

Sandra Yucra, M.Sc.,[a,b] *Manuel Gasco, M.Sc.,*[a,b] *Julio Rubio, B.Sc.,*[a,b] *Jessica Nieto, B.Sc.,*[a] *and Gustavo F. Gonzales, M.D., D.Sc.*[a,b]

[a] Department of Biological and Physiological Sciences, Faculty of Sciences and Philosophy, and [b] Instituto de Investigaciones de la Altura, Universidad Peruana Cayetano Heredia, Lima, Peru

Objective: To evaluate the effect of different fractions of Black Maca (*Lepidium meyenii*), obtained from the hydroalcoholic extract, on spermatogenesis.
Design: Animal study.
Setting: Animal and laboratory facilities at a university.
Animal(s): Forty two adult male rats from the Holtzman strain (3 months old).
Intervention(s): Hydroalcoholic extract of Black Maca was partitioned with the following solvents: petroleum ether, chloroform, ethyl acetate, n-butanol, and water to obtain each fraction. Forty-two rats were divided in different groups according the fraction administered and vehicle. The hydroalcoholic extract of Black Maca and its fractions and vehicle were given orally by gavage for 7 days.
Main Outcome Measure(s): Daily sperm production, epididymal sperm count, and sperm count in the vas deferens.
Result(s): Daily sperm production was higher in the ethyl acetate group compared with all other groups. The epididymal sperm count was higher in rats treated with ethyl acetate fraction compared with rats treated with vehicle (control), petroleum ether, n-butanol, or water fractions. The sperm count in vas deferens was lower in rats treated with ethyl acetate, petroleum ether, or water fractions compared with the control group; thus, the sperm count in vas deferens in rats treated with chloroform and n-butanol fractions was higher than in the petroleum ether group.
Conclusion(s): The greatest effect on spermatogenesis was observed in the ethyl acetate fraction from the hydroalcoholic extract of Black Maca, suggesting that the compounds related to the beneficial effect on sperm production of Black Maca are presented in this fraction. Antioxidant components could play a role in the effect of increased epididymal sperm concentration observed in the model. (Fertil Steril® 2007;■:■-■. ©2007 by American Society for Reproductive Medicine.)

Key Words: Spermatogenesis, Black Maca, fractions, *Lepidium meyenii*

Lepidium meyenii Walp., a cruciferous vegetable known as Maca, grows exclusively between 4000 and 4500 m above sea level in the central Peruvian Andes, particularly in Junin plateau. Anecdotal evidence claims that this plant enhances fertility (1), and previous experimental studies have demonstrated the traditional fertility-enhancing properties of the hypocotyls of Maca in animal models (2–6) and in humans without affecting the levels of serum testosterone, luteinizing hormone (LH), or follicle stimulating hormone (FSH) (7).

Different varieties of Maca have been described according to the color of its hypocotyls (8, 9). Previous studies have demonstrated that Red, Yellow, and Black Maca show differences in their nutritional components (9). For instance, Red Maca has a higher content of pure protein and potassium, and lower content of soluble direct reducing sugars, riboflavin, and iron than Black Maca, whereas Yellow Maca has intermediate values for these compounds (9). Accordingly, different biologic properties have been observed among the black, yellow, and red varieties of Maca (10, 11). For instance, there is evidence that the black variety of Maca has more beneficial effects on spermatogenesis (11) and learning (12) than the yellow or red variety. Red Maca can reduce prostate size in rats with experimentally induced benign prostate hyperplasia (10).

Because previous studies have found Black Maca to have the greatest effect on sperm production when compared with Yellow and Red Maca, we elucidated the fraction responsible for this effect by assessing the following parameters in the adult male rat: daily sperm production

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Reprint requests: Sandra Yucra, M.Sc., Universidad Peruana Cayetano Heredia, Biological and Physiological Sciences, P.O. Box 1843, Lima, Lima 31, Peru (FAX: 00-51-1-4821195; E-mail: 23411@upch.edu.pe).

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FLA 5.0 DTD ■ fns22298 ■ 10 July 2007 ■ 3:07 am ■ ce 21

(DSP), epididymal sperm count, and vas deferens sperm count.

MATERIALS AND METHODS

Plant Material

The dried hypocotyls of Black Maca were collected in Carhuamayo, Junin, at 4000 m above sea level. The biologic activity of the plant is located in the hypocotyls, which the area's residents consume after natural drying (11). The plant was authenticated by Irma Fernandez, a botanist in the Department of Pharmaceutical Sciences, Universidad Peruana Cayetano Heredia. The voucher number IFV 2374 was deposited at the department.

Preparation Hydroalcoholic Extract of Black Maca

The hydroalcoholic extract of Black Maca was prepared with aqueous ethanol (60%; *v*/*v*) by percolation at room temperature for 24 hours, and concentrated at low pressure to constant weight. The extract was prepared by Eng. Alfonso Higa from Agroindustrial Chanchamayo (Lima, Peru). A sample with 100 grams of dried Black Maca hypocotyls produced 7.6 g of hydroalcoholic Black Maca. This extract was kept in a refrigerator at 4°C until use.

Extraction

The hydroalcoholic extract of Black Maca (30 g) was suspended in 50 mL of water and was successively partitioned three times (60 mL/each) with different solvents in the following order: petroleum ether (Tedra Company INC, OH), chloroform (Fisher Scientific, Fair Lawn, NJ), ethyl acetate (Mallinckrodt Baker, Inc., Paris, KY), and n-butanol (Merck KGaA, Darmstadt, Germany). The resulting extracts were evaporated under vacuum to afford the following fractions: petroleum ether (23.7 mg), chloroform (176.8 mg), ethyl acetate (207.7 mg), n-butanol (2.46 g), and the remaining aqueous solution (which will be called the water fraction) (25.93 g). Fractions with different polarities were created by successive extraction with solvents with different polarities in an increasing manner.

Animals

A total of 42 adult male rats from the Holtzman strain (3 months old) were obtained from the animal house of the Universidad Peruana Cayetano Heredia (Lima, Peru). The rats were divided randomly into seven groups ($n = 6$) according to the treatment: hydroalcoholic extract and petroleum ether, chloroform, ethyl acetate, n-butanol, or aqueous fraction. The control group received distilled water or 1% dimethyl sulfoxide (DMSO, J.T. Baker Inc., Phillipsburg, NJ) as vehicle. Rats were housed at six per cage and were maintained under controlled conditions at 22°C with a 12/12 hours light/dark cycle in the animal house at the Universidad Peruana Cayetano Heredia. Rats were provided with food and water ad libitum.

Experimental Design

The rats were treated with 2.25 mg of petroleum ether, 16.79 mg of chloroform, or 19.73 mg of ethyl acetate fraction diluted in 45 mL of distilled water with 1% DMSO. In addition, 0.23 g of n-butanol and 2.46 g of aqueous fractions were diluted in 45 mL of distilled water. Also, the hydroalcoholic extract (2.85 g) was diluted in distilled water (45 mL). The 1 g/kg body weight doses were chosen based on the preliminary studies developed in our laboratory. There were no differences between rats treated with DMSO or distilled water (data not shown); for this reason, the data obtained in both groups were pooled.

The treatments were administered by oral route. A no. 18 intubation needle (Fisher Scientific, Pittsburgh, PA) was used to administer 1 mL of each solution. Rats were killed on day 8 by excess inhalation of ether. The animals were treated according to the standards of the U.S. National Institutes of Health for the care and use of laboratory animals (13). The institutional review board of the Scientific Research Office of the Universidad Peruana Cayetano Heredia approved the study (SIDISI-UPCH: 52208).

Body and Organ Weights

The body weight change of each group was defined as the difference between initial and final body weights. After that animals were killed (24 hours after the final treatment), selected organs (testes, epididymis, seminal vesicles, and ventral prostate) were carefully dissected out, cleaned of adhering connective tissue, and accurately weighed.

Daily Sperm Production

After the left testis had been thawed, the capsule was detached, and the parenchyma was homogenized in 10 mL of 0.9% saline-0.05% (*v*/*v*) Triton X-100 solution for 1 minute by a homogenizer (14). After a dilution of 1/10, the number of homogenization-resistant elongated spermatid nuclei per testis was determined with a hemocytometer. Counts for four hemocytometer chambers were averaged. The DSP and its efficiency (DSP per gram of testis) were determined by division of the elongated spermatid count per testis and spermatids per gram of testis by 6.3, the duration of steps 17 to 19 spermatids in the seminiferous epithelial cycle for Holtzman rats (14, 15). The epididymal sperm transit rate was calculated by dividing the cauda epididymal sperm number by the DSP (16).

Epididymal Sperm Count

Homogenization-resistant epididymal sperms from 42 nonperfused rats were counted as previously described elsewhere (17) with some modifications. In brief, caput and corpus epididymis were cut and homogenized separately to the cauda epididymis. Homogenization was performed in 5 mL of saline (NaCl 0.9%). Homogenates were kept refrigerated at 4°C for 24 hours to allow sperm to be released from the walls. Then 10 μL of the refrigerated homogenate was added to 70

FLA 5.0 DTD ■ fns22298 ■ 10 July 2007 ■ 3:07 am ■ ce 21

μL of eosin (2%), and a sample was placed in a Neubauer chamber. Head sperms were counted in 25 squares for four times. The average sperm count of each rat were multiplied by 0.06 (sperm × 10^6/mL) and then by 5 mL (sperm × 10^6 per caput/corpus or cauda). Data are referred as sperm per caput/corpus or cauda epididymis.

Vas Deferens Sperm Count

The vas deferens was dissected in two parts, one corresponding to the proximal end and the second to the distal end. Each part was homogenized with 1 mL of saline. An aliquot was diluted with two parts of eosin (0.2%). Homogenization-resistant sperm heads were counted in the 25 squares of the Neubauer chamber. Four chambers were measured in each sample, and they were averaged. Results from each part (proximal or distal end) were multiplied by 0.03 and defined as sperm × 10^6 per part of vas deferens. Data were expressed as the total amount of sperms in vas deferens (sperm count in proximal end + sperm count in distal end).

Statistical Analysis

Data were analyzed using the statistical package stata (v. 8.0) for PC (Stata Corporation, College Station, TX).

Data are presented as mean ± SEM. Homogeneity of variances was assessed by the Bartlett test. If variances were homogeneous, differences between groups were assessed by analysis of variance (ANOVA). If F-value in the ANOVA test was significant, the differences between pair of means were assessed by the Scheffé test.

When variables were not homogeneous, the Kruskal–Wallis test was used to assess differences between groups. If the result was statistically significant, the differences between the pair of medians were assessed by the Mann–Whitney U-test. A value of $P<.05$ was considered to be statistically significant.

RESULTS

Body and Organ Weights

Table 1 shows the effect of different fractions of Black Maca on body weight changes and relative reproductive organ weights.

At the end of each treatment, the final body weight increased in all groups as follows: 8.24%, 8.02%, 6.30%, 5.88%, 4.87%, 4.43%, and 6.76% for rats treated with vehicle (control), hydroalcoholic extract, petroleum ether, chloroform, ethyl acetate, n-butanol, and aqueous fractions, respectively. No differences in body weight were observed among groups at the end of treatment (not statistically significant).

Regarding to relative reproductive organ weight, no statistically significant differences were observed in rats treated with hydroalcoholic extract of Black Maca and its fractions compared with control group. Also, no statistically significant differences were observed between rats treated with any fraction of hydroalcoholic extract of Black Maca.

Daily Sperm Production

The rats treated with ethyl acetate had higher levels in DSP, its efficiency (DSP/g. testis), and sperm transit compared with all groups after 7 days of treatment ($P<.05$). Taking into account DSP/testis, no difference was observed in the control group compared with rats treated with hydroalcoholic extract and petroleum ether, chloroform, n-butanol, or aqueous fractions (not statistically significant; Fig. 1). Similar values were observed with data from the DSP. In sperm transit, there were no statistically significant differences among the groups (data not shown).

Sperm Count in Epididymis and Vas Deferens

Figure 2 shows the effect on epididymal sperm count in adult rats of the 7 days of treatment with hydroalcoholic extract of Black Maca and its fractions. An increase in total epididymal sperm count was observed in rats treated with petroleum

TABLE 1

Effect of different fractions of Black Maca on body weight changes and relative reproductive organ weights.

		Relative weights			
	Increase in body weight (g)	Left testis	Left epididymis	Seminal vesicle	Ventral prostate
Control group	23.40 ± 2.87	0.48 ± 0.02	0.16 ± 0.01	0.35 ± 0.04	0.11 ± 0.01
Petroleum ether	20.60 ± 11.52	0.48 ± 0.01	0.14 ± 0.01	0.37 ± 0.01	0.11 ± 0.01
Chloroform	18.75 ± 1.65	0.49 ± 0.01	0.15 ± 0.01	0.31 ± 0.02	0.12 ± 0.01
Ethyl acetate	18.80 ± 2.56	0.48 ± 0.01	0.15 ± 0.01	0.34 ± 0.02	0.10 ± 0.02
n-butanol	15.17 ± 1.71	0.45 ± 0.02	0.13 ± 0.01	0.30 ± 0.01	0.10 ± 0.01
Water or aqueous	20.63 ± 2.47	0.48 ± 0.02	0.14 ± 0.01	0.29 ± 0.01	0.10 ± 0.01
Hydroalcoholic extract	23.00 ± 7.13	0.51 ± 0.02	0.15 ± 0.01	0.32 ± 0.01	0.11 ± 0.01

Note: No statistically significant differences among groups.

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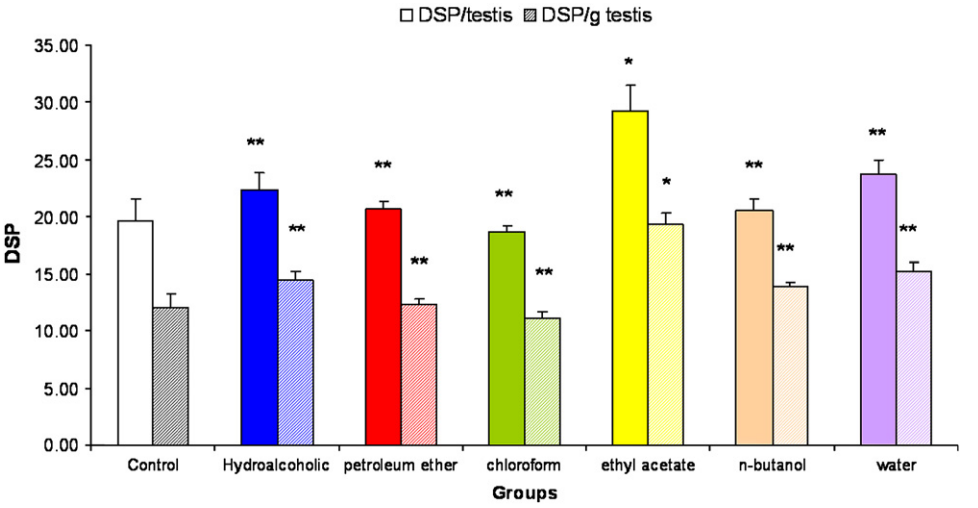
FIGURE 1

Effect of different fractions of Black Maca on daily sperm production (DSP/testis) and its efficiency (DSP/gram of testis). Data are mean ± SEM of six rats per group. Differences between groups were assessed with Mann-Whitney *U* test. **P*<.05 with respect to the control group. ***P*<.05 with respect to ethyl acetate fraction.



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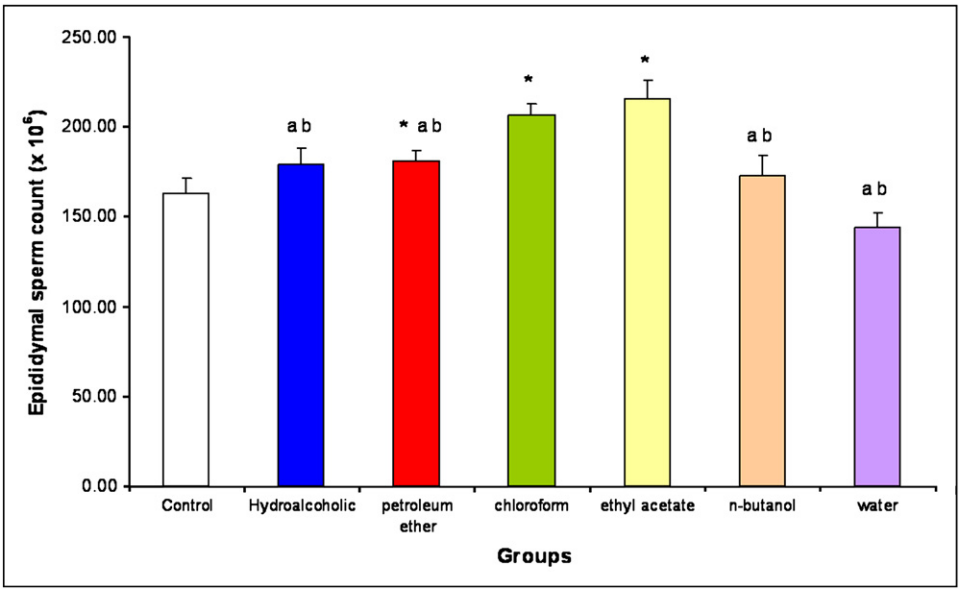
ether (181.25 ± 5.30 × 10^6 sperm per epididymis; *P*<.05), chloroform (206.32 ± 6.57 × 10^6 sperm per epididymis; *P*<.05), and ethyl acetate fractions (215.72 ± 10.28 × 10^6 sperm per epididymis; *P*<.05) compared with the control group (163.27 ± 7.96 × 10^6 sperm per epididymis). The greatest effect was observed in rats treated with ethyl acetate

FIGURE 2

Effect of different fractions of Black Maca on epididymal sperm count. Data are mean ± SEM of six rats per group. Differences between groups were assessed with Mann-Whitney *U* test. **P*<.05 with respect to the control group. [a]*P*<.05 with respect to chloroform fraction. [b]*P*<.05 with respect to ethyl acetate fraction.



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fraction. In addition, rats treated with ethyl acetate or chloroform fractions showed a higher sperm number in epididymis than rats treated with hydroalcoholic extract (179.25 ± 8.89 × 10^6 sperm per epididymis; $P<.05$), petroleum ether, n-butanol, and aqueous fractions (181.25 ± 5.30, 172.48 ± 11.45, and 144.17 ± 8.08 × 10^6 sperm per epididymis, respectively; $P<.05$). No statistically significant differences were found when the control group was compared with the hydroalcoholic extract group.

Figure 3 shows the sperm count in vas deferens after 7 days of treatment with hydroalcoholic extract of Black Maca and its fractions. Rats treated with ethyl acetate (4.60 ± 0.40 × 10^6 sperm/vas deferens), petroleum ether (4.65 ± 0.39 × 10^6 sperm per vas deferens), and aqueous fraction (5.1 ± 1.38 × 10^6 sperm per vas deferens) showed lower levels in sperm count in vas deferens compared with the control group (9.59 ± 2.11 × 106 sperm per vas deferens; not statistically significant). There were no statistically significant differences between the control group and hydroalcoholic extract group (9.05 ± 1.38 x 106 sperm per vas deferens). The sperm count in rats treated with chloroform fraction (8.88 ± 1.79 × 10^6 sperm per vas deferens) was higher than in those treated with petroleum ether and ethyl acetate fractions ($P<.05$). Rats treated with n-butanol fraction (7.77 ± 0.58 × 10^6 sperm per vas deferens) showed an increase in sperm number in vas deferens when compared with those treated with petroleum ether fraction ($P<.05$).

DISCUSSION

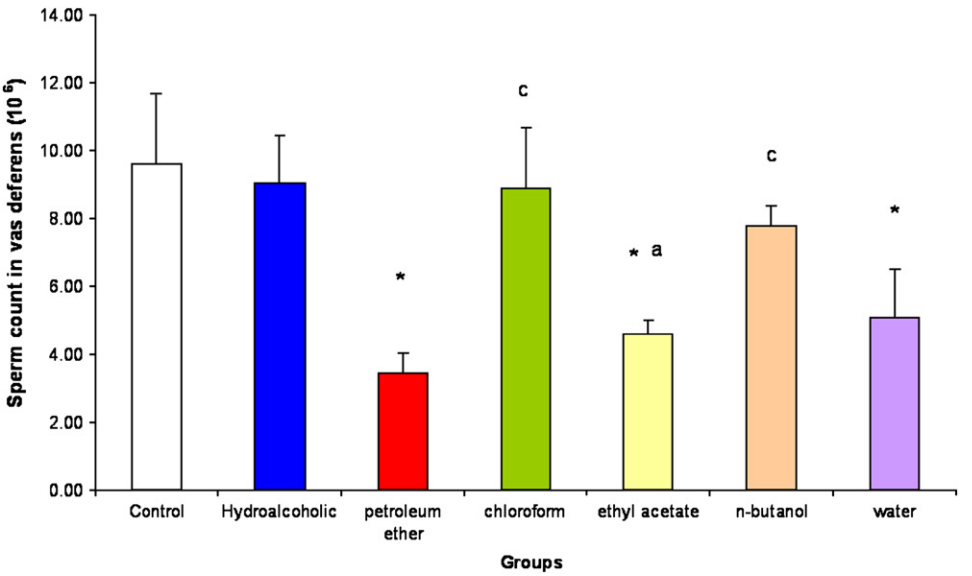
Maca can be found in several varieties determined by external color (18). Previous studies have shown that there are differences in the biologic effect of three varieties of Maca: Red, Yellow, and Black (10–12). Indeed, Black Maca appears to have the greater beneficial effect on sperm count and motility after 42 days of treatment (11). Moreover, it was demonstrated that Black Maca increased DSP after as few as 7 days of treatment (17). For this reason, we elucidated the fraction from the hydroalcoholic extract of Black Maca that presented greatest effect on sperm production.

Previous studies have demonstrated that oral administration of aqueous extract of Black Maca can positively affect DSP and epididymal sperm count (17). In our study, oral treatment with ethyl acetate fraction of Black Maca for 7 days had the most beneficial effect on epididymal sperm count and DSP compared with other fractions.

It also has been suggested that Black Maca can modulate processes related to DSP and sperm count in epididymis (17). The epididymis, a natural sperm reservoir, has maturational and storage functions, and it protects spermatozoa from oxidative injury by encouraging scavengers of reactive oxygen species (19). It has been observed that rats treated for 8 weeks with ascorbic acid, a potent antioxidant, showed a significantly increased epididymal sperm concentration ($P<.05$) when compared with control animals (20). The administration

FIGURE 3

Effect of different fractions of Black Maca on sperm count in vas deferens of adult male rats. Data are mean ± SEM of six rats per group. Differences between groups were assessed with Mann-Whitney *U* test. *$P<.05$ with respect to the control group. [a]$P<.05$ with respect to chloroform fraction. [c]$P<.05$ with respect to petroleum ether fraction.



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of melatonin or vitamin E can prevent adverse effects of homocysteine on plasma antioxidant enzyme activities, testosterone level, epididymal sperm count, and motility in male rats (21). In the male rabbit, treatment with isoflavones resulted in an increase in sperm count and antioxidant activity in seminal plasma (22). Moreover, when piperine, an alkaloid present in the fruits of Black Pepper (*Piper migrum*), is administered to adult rats, there is a decrease in the activity of antioxidant enzymes in the epididymis and reduced epididymal sperm count (23). Consistent with this notion, several investigators had demonstrated that Maca has antioxidant properties (24–26).

It has been reported that reactive oxygen species, generated in vitro, can cause DNA fragmentation in human sperm, but that this could be prevented by preincubation with antioxidants (27). Moreover, pretreatment in vitro with tocopherol and ascorbic acid prevented sperm DNA damage induced by irradiation (28). Previous studies have demonstrated that ethyl acetate fractions, among others, contain phenolic constituents (29). In addition, it has been demonstrated that these constituents have potent scavenging activity (29). Again, other investigators found an increase in total phenolic content when compared with an ethanol extract and its ethyl acetate fraction (27), supporting the hypothesis that the effect observed on sperm production might be related to these compounds and their antioxidant activity.

Previous phytochemical investigations have shown the presence of phenolic compounds such as flavonoids (9), flavonol, and quercetine (30) in Maca. In fact, Maca has been demonstrated to have antioxidant properties in vitro and in vivo (24, 25). For instance, lead acetate administration causes an increase in oxidative status, and it has been shown in male rats to reduces DSP and epididymal sperm count; Maca reversed this effect (6). Thus, Black Maca could play a role in regulating sperm number by maintaining the balance between oxidant and antioxidant status.

Ethyl acetate fraction from hydroalcoholic extract of Black Maca increased the DSP and epididymal sperm count at 63.2 $\times$ 10^6 per day in rats. It is likely that this fraction has some secondary metabolites as phenols that provide it with this property. The specific active ingredients in Black Maca have yet to be identified and purified. Further studies are necessary to elucidate which compounds of the ethyl acetate fraction are the source of the property that enhances spermatogenesis in rats.

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FLA 5.0 DTD ■ fns22298 ■ 10 July 2007 ■ 3:07 am ■ ce 21

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1 **Effect of different fractions from hydroalcoholic extract of Black Maca (*Lepidium meyenii*) on testicular function in adult male rats**

S. Yucra, M. Gasco, J. Rubio, J. Nieto, and G. F. Gonzales
Lima, Peru

The greatest effect on spermatogenesis in adult male rats was observed in the ethyl acetate fraction from the hydroalcoholic extract of Black Maca (*Lepidium meyenii*), suggesting that the compounds related to the beneficial effect on sperm production of Black Maca are presented in this fraction. Antioxidant components could play a role in the effect of increased epididymal sperm concentration observed in the model.

FLA 5.0 DTD ■ fns22298 ■ 10 July 2007 ■ 3:07 am ■ ce 21