J Nat Med (2007) 61:452–457
DOI 10.1007/s11418-007-0173-4

NOTE

Effect of terpenoidal fraction of *Echinops echinatus* roots on reproductive parameters of male rats

S. A. Padashetty · S. H. Mishra

Received: 26 February 2007 / Accepted: 21 May 2007 / Published online: 24 July 2007

Abstract The present study was undertaken to evaluate the effect of terpenoidal fraction prepared from the petroleum ether extract of the roots of *Echinops echinatus* on male reproductive parameters. The studies were carried out at two different dose levels of 30 and 60 mg/kg body weight using Wistar albino rats. Treatment with terpenoidal fraction showed a decrease in the relative weight of the reproductive organs without affecting the final body weight of the animals, and a significant decrease ($P < 0.01$) in serum testosterone levels and cauda epididymal sperm concentration compared with animals in the control group. Histology of the testis in the control group showed normal features with successive stages of transformation of the seminiferous epithelium into spermatozoa, whereas an acute treatment with two different doses of terpenoid fraction showed a significant reduction ($P < 0.01$) in the seminiferous tubular diameter and germinal epithelial cell thickness. Thus, it was observed that the acute treatment with terpenoid fraction of the *E. echinatus* roots to male rats exhibits antifertility properties by interfering with the reproductive function of the testis. The fraction was standardised in terms of its marker (lupeol) content using high-performance thin-layer chromatography (HPTLC).

Keywords *Echinops echinatus* · Antifertility · HPTLC · Seminiferous tubules · Terpenoids · Lupeol

S. A. Padashetty · S. H. Mishra (✉)
Pharmacy Department, Faculty of Technology and Engineering, Kalabhavan, The M. S. University of Baroda, Vadodara 390 001 Gujarat, India
e-mail: shmishra48@rediffmail.com

Introduction

Echinops echinatus Roxb (Compositae) is a pubescent annual herb of 1–3 feet in height, being distributed throughout India [1]. Roots of this plant are sold in different Indian markets under the trade name of brahmadandi [2] and are employed in the treatment of disorders related to the reproductive system, such as abortifacients, to hasten the process of delivery, in urinary discharges, as a diuretic and in syphilis [3–6]. Experimental studies in female rats showed that the antifertility effects of *E. echinatus* roots and its various extracts are due to antiestrogenic activity of the plant [7]. Chaturvedi et al. [8] reported that oral administration of crude alcohol extract of *E. echinatus* roots to male rats at the dose of 200 mg/kg body weight resulted in reversible antifertility effects, with no adverse toxic effects. In our continuous investigations on the chemical constituents and biological activities of *E. echinatus*, we found the presence of terpenoids as the chief constituents in the plant; therefore, it was thought worthwhile to prepare a fraction containing terpenoids and investigate its effect on the male reproductive parameters in Wistar albino rats. The terpenoidal fraction was standardised in terms of its marker (lupeol) content using high-performance thin-layer chromatography (HPTLC).

Materials and methods

Plant material

Roots of *E. echinatus* were collected in the month of August 2005 from the outfield of Gulbarga City, Karnataka, India, and were authenticated in the Botany Department of The M. S. University of Baroda, Vadodara, India. A voucher specimen (no. Pharmacy/EE/05-06/01/SP) has been

deposited in the Pharmacy Department of The M. S. University of Baroda, Vadodara, India.

Preparation of extract and terpenoidal fraction

A coarse powder (500 g) of the shade-dried roots was extracted with petroleum ether (60/80) in a Soxhlet apparatus. The extract was concentrated on a rotary vacuum evaporator (BUCHI Rotavapor R 200) and dried under vacuum (yield = 3.96% w/w). Vacuum-dried petroleum ether extract was subjected to saponification, and the unsaponifiable material rich in terpenoids was separated out and dried under vacuum (25% w/w) [9]. Vacuum-dried terpenoid-rich fraction of petroleum ether extract (TRFPE) was taken for further studies.

Chemical test and quantitative estimation of the marker compound by HPTLC

Obtained TRFPE was subjected to Liebermann Burchard test for terpenoids [10]. In brief, a small quantity of the TRFPE was dissolved in chloroform and treated with acetic anhydride and few drops of concentrated sulfuric acid, which gives a dark pink to red colour, indicating the presence of terpenoids, which were further separated on a thin-layer chromatography (TLC) plate and detected by treatment with anisaldehyde sulfuric acid reagent.

HPTLC conditions

The HPTLC system (CAMAG) consisting of a LINOMAT-IV applicator and TLC Scanner-III, precoated plates of silica gel G_{60} F_{254} (E-Merck) and a mobile phase containing toluene:chloroform:ethyl acetate:acetic acid (10:2:1:0.1) were used in the studies. Detection was done using antimony trichloride reagent [11] followed by scanning under ultraviolet (UV) light at 366 nm.

Preparation of sample solutions

A test solution containing 1 mg/ml of the TRFPE was prepared in chloroform and methanol (1:1), and similarly, a stock solution of the pure lupeol (obtained commercially from Sigma Chemicals, USA) was prepared by dissolving 10 mg of accurately weighed pure lupeol in 10 ml of chloroform and methanol (1:1); required concentrations were then obtained by further dilution of the stock solutions.

Calibration curve

The standard solution (20–100 ng per spot) was applied on a precoated TLC plate of uniform thickness. The plate was developed to a height of 8 cm using the proposed solvent system in a twin-trough chamber saturated previously with vapours of the mobile phase for not less than 20 min. The developed plates were treated with antimony trichloride and heated at 110°C for 10 min and then scanned densitometrically at 366 nm. The peak area was recorded and the calibration curve prepared by plotting peak area versus concentration of lupeol.

Estimation of the lupeol in TRFPE

Next, 10 µl of the test solution (200 ng per spot) was applied in triplicate on a precoated TLC plate of uniform thickness. The plate was developed in the solvent system and the peak area of lupeol was recorded as described above for the calibration curve. The amount of lupeol present in the sample was calculated from the calibration curve of lupeol.

Animals

Adult albino Wistar rats 90 days old and weighing between 210 and 270 g were obtained from M/s Zydus Cadilla Research Centre, Ahmedabad, India, and housed at a temperature of 24–28°C with a relative humidity of 45–55% with free access to food and water ad libitum. Before the experiment began, the local committee of ethics on animal experimentation approved all experimental procedures (no. 404/01/a/CPCSEA).

Acute toxicity studies

Healthy adult albino (Wistar) rats of either sex, starved overnight, were divided into four groups ($n = 6$) and were fed with increasing doses (50, 100, 200 and 300 mg/kg body weight) of the TRFPE. The animals were observed up to 14 days for toxicity and mortality. Finally, 1/10th of the highest tolerable dose was taken for further studies [12].

Treatment

Male albino Wistar rats were used in the studies and randomly divided into three groups ($n = 8$): group 1 received the vehicle (1 ml/day of 1% tween 80 in water) orally for 8 days and served as control. Groups 2 and 3 were administered TRFPE suspended in 1% tween 80 at a dose of 30 and 60 mg/kg body weight, respectively, for a period of 8 days.

Effect on body and organ weight

The initial and final body weights of the animals were recorded, and the change in body weight was determined.

The animals were sacrificed by cervical dislocation method, and the sex organs (testis, epididymis, vas deferens and seminal vesicles) were dissected out carefully, freed from adhering tissues and weighed to the nearest 0.1 mg.

Epididymal sperm count

Homogenisation-resistant epididymal sperm were counted as described by Bustos-Obregon and Gonzalez-Hormazabal [13] with some modifications. Homogenisation was performed in 5 ml of 0.9% saline, and the homogenised epididymal preparation was refrigerated at 4°C for 24 h to allow sperm to be released from the walls. Data are referred to as sperm (10^6) per epididymis.

Serum testosterone

Blood samples were collected simultaneously during sacrification of animals, and serum samples were separated and stored according to procedures given by the National Committee for Clinical Laboratory Standards (NCCLS) [14]. Serum levels of testosterone were assayed by a competitive immunoassay using direct chemiluminescent technology on an autoanalyser (ADIVA centaur) at Endocrine Laboratory and Invitro Allergy Testing Centre, Ahmedabad, India.

Testicular histomorphology

Testis from each group was excised quickly during the dissection of animals and fixed in 10% buffered neutral formalin. Ultrathin sections of the testicular tissue were cut and stained with hematoxylin and eosin. Histological examinations included the mean seminiferous tubular diameter (STD) and germinal epithelial cell thickness (GECT). Three slides from the upper, lower and mid portions of the testis were prepared and evaluated for each testis. The mean STD and GECT were determined in 20 seminiferous tubules of each section using a projection microscope [15], and the values were expressed in terms of micrometers (μm). Microphotographs were made using an Olympus BX 40 microscope attached to an Olympus DP12 digital camera.

Data analysis

To determine statistically significant differences among treatment groups, data were analysed by using one-way analysis of variance (ANOVA) followed by Dunnet test. Values of $P < 0.05$ were considered statistically significant.

Results

Preliminary chemical examination of TRFPE showed an intense pink colour with the Liebermann Burchard test, indicating rich terpenoid content in the plant, which was then detected on a TLC plate by treatment with anisaldehyde sulfuric acid reagent (Fig. 1). Under the chromatographic conditions described above, the relationship between the concentration of lupeol and the peak response was linear, with a correlation coefficient of 0.994 (Fig. 2),

Fig. 1 Detection of terpenoids on thin-layer chromatography (TLC) using anisaldehyde sulfuric acid reagent

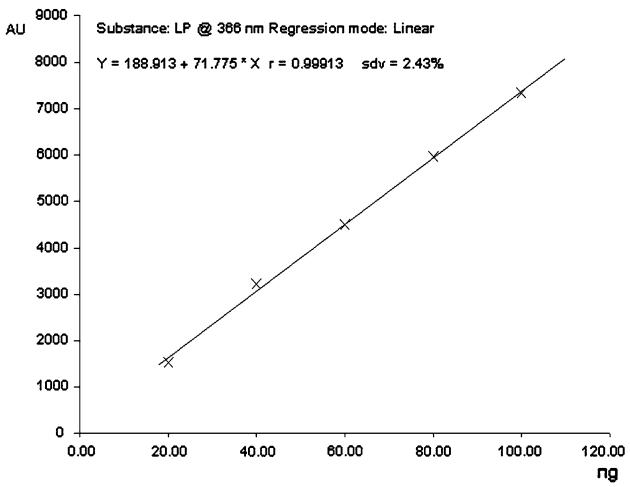

Fig. 2 Linearity of the calibration curve for quantification of lupeol in terpenoid-rich fraction of petroleum ether extract (TRFPE)

and the R_f of lupeol was found to be 0.52. The chromatogram of TRFPE showed the presence of six peaks at R_f values 0.12, 0.25, 0.36, 0.52, 0.74 and 0.99, of which lupeol was detected at R_f 0.52. The content of lupeol in the TRFPE was found to be 38.3% w/w.

Results of acute toxicity studies showed that TRFPE, when administered orally at the maximum dose of 300 mg/kg body weight, did not produce any evident sign of toxicity or mortality. The final body weights of animals in both groups were increased compared with their respective initial weights; however, the increase was found to be greater in the control group compared with the treated groups. On the other hand, a decrease in relative weights of testis, cauda epididymides and seminal vesicles were observed in the treated group compared with the control group (Table 1).

Treated animals exhibited a significant decrease in serum testosterone levels and cauda epididymal sperm concentration compared with animals in the control group (Table 2). Histology of the testis in the control group showed normal features with successive stages of transformation of the seminiferous epithelium into spermatozoa, whereas histological examination of the testis after acute treatment with two different doses of TRFPE at 30 and 60 mg/kg body weight showed the inhibitory effects on the growth of seminiferous epithelium and on spermatogenesis (Fig. 3), with a significant reduction in the STD and GECT (Fig. 4; Table 2).

Discussion

Increase in the final body weight of the animals suggests that the treatment of rats with TRFPE did not induce any overtoxicity to the animals. A significant decrease in testicular weight was observed in the animals treated with TRFPE, which is known to be related to the number of spermatids and spermatozoa in the tissue [16]. Observation of the testicular histomorphology showed that the extract inhibits the growth of seminiferous epithelium and consequently a decrease in seminiferous tubular diameter. It is probable that the observed effects on testis induced by the *E. echinatus* extract were due to the significant decrease in testosterone level, which is responsible for the diminished spermatogenesis, and hence is reduction in sperm counts [17, 18]. Circulating levels of testosterone are required for the maintenance of normal structure and function of the accessory sex organs, though the threshold levels may vary with organs. Reduced epididymal sperm concentration and weight of the accessory sex organs further supports the suppressed concentration of testosterone in the circulation [19, 20].

The results are further supported by the fact that plants containing triterpenoids, especially those of the lupane group including pure lupeol acetate exhibit antifertility effects in male rats [21, 22]. Preliminary phytochemical screening revealed the presence of terpenoids as the chief constituents in the TRFPE. During the phytochemical investigations, we detected the presence of lupeol in the roots of *E. echinatus*,

Table 1 Change in the body weight and weight of reproductive organs after terpenoid-rich fraction of petroleum ether extract (TRFPE) treatment in male rats

Groups (n = 8)	Body weight (g)			Weight of male reproductive organs mg/100 g body weight (mean ± SEM)			
	Initial	Final	Increase (%)	Testis	Epididymis	Vas deferens	Seminal vesicles
Control (vehicle)	276.2 ± 15.6	286.9 ± 22.6	3.8	542.2 ± 25.3	235.8 ± 12.9	57.7 ± 5.3	252.9 ± 20.7
TRFPE (30 mg/kg body weight)	246.6 ± 11.9	252.9 ± 13.4	2.5	598.9 ± 24.1	217.2 ± 16.5	55.2 ± 5.5	179.6 ± 16.2*
TRFPE (60 mg/kg body weight)	218.6 ± 19.4	226.6 ± 22.7	3.6	526.7 ± 25.8	206.6 ± 09.3	60.1 ± 6.2	245.8 ± 15.6

Values are mean ± SEM (n = 8)

*$P < 0.05$ versus control

Table 2 Effect of oral administration of terpenoid-rich fraction of petroleum ether extract (TRFPE) on testicular histomorphology and epididymal sperm concentration

Groups (n = 8)	Seminiferous tubular diameter (μm)	Germinal epithelial cell thickness (μm)	Sperm count 10^6/epididymis	Testosterone (ng/ml)
Control (vehicle)	321.1 ± 8.8	87.4 ± 1.6	175.0 ± 11.5	3.4 ± 0.5
TRFPE (30 mg/kg b. wt)	253.0 ± 4.5**	63.6 ± 2.0**	102.1 ± 5.1**	1.4 ± 0.2**
TRFPE (60 mg/kg b. wt)	266.9 ± 4.2**	60.0 ± 2.3**	110.0 ± 10.0**	1.4 ± 0.3**

Values are mean ± SEM (n = 8)

*$P < 0.05$ versus control; **$P < 0.01$ versus control

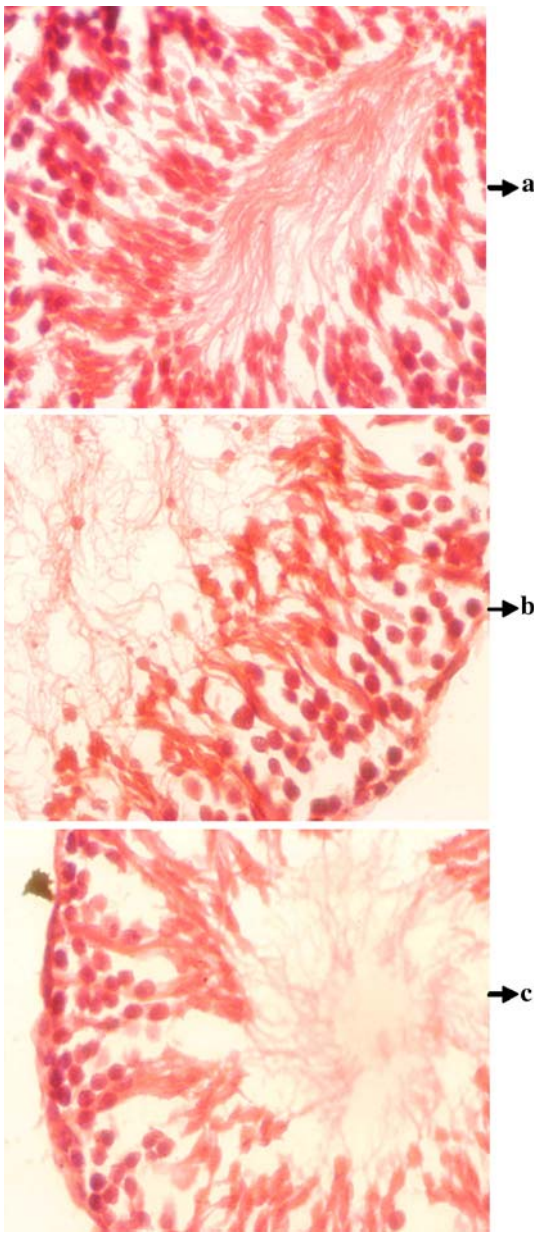

Fig. 3 Histology of the testis in different groups. **a** Control. **b** Terpenoid-rich fraction of petroleum ether extract (TRFPE) 30 mg/kg. **c** TRFPE 60 mg/kg showing successive stages of transformation of the seminiferous epithelium into spermatozoa (×40)

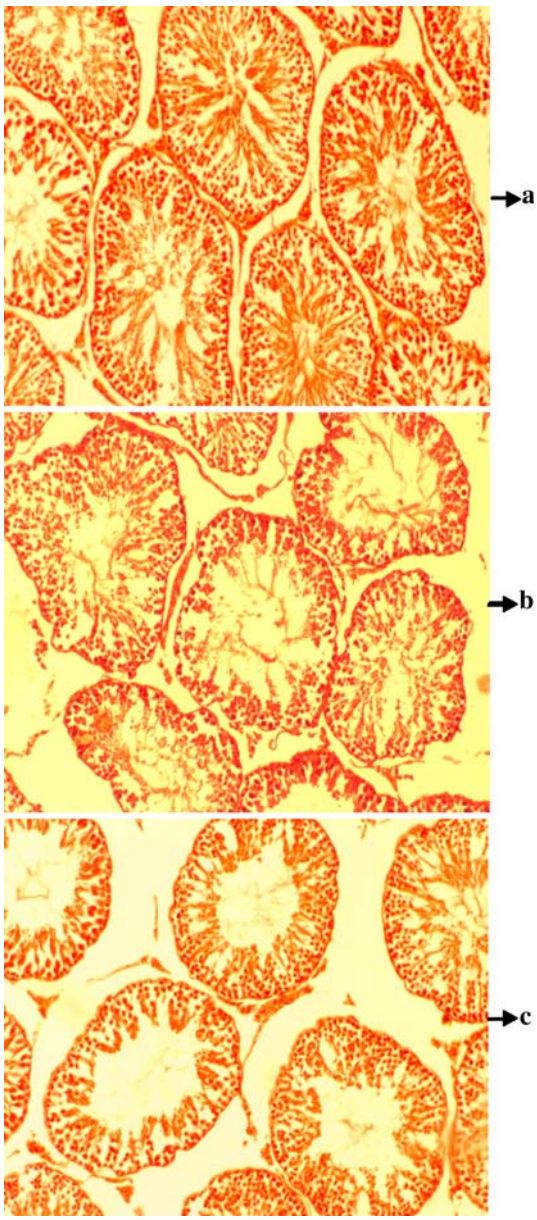

Fig. 4 Effect of terpenoid-rich fraction of petroleum ether extract (TRFPE) on seminiferous tubular diameter and germinal epithelial cell thickness of different groups. **a** Control. **b** Treated with TRFPE 30 mg/kg. **c** TRFPE 60 mg/kg (×10)

and it has also been reported to contain taraxasterol acetate [23], a pentacyclic triterpenoid of the lupane skeleton. The observed activity may thus be due to a rich content of terpenoids, mainly those of the lupane type, in the roots of the plant *E. echinatus*.

Conclusions

In conclusion, acute treatment with TRFPE of the *E. echinatus* roots to male rats was found to exhibit antifertility properties by interfering with the reproductive function of the testis, which may be due to the major class of chemical constituents (viz., triterpenoids of the lupane group) in the extract. Further investigations on characterisation of chemical constituents and more clear mechanisms may result in justifying this plant as a safe and effective therapeutic agent for regulation of male fertility.

Acknowledgments One of the authors Mr. Somashekar Padashetty would like to thank All India Council for Technical Education (AICTE), New Delhi, for providing the financial assistance in the form of a National Doctoral Fellowship to carry out this work.

References

1. Anonymous (1952) The wealth of India, raw materials, vol 3. CSIR, New Delhi p 127
2. Issar RK (1974) The botanical identification of market sample of brahmadandi. J Res Ind Med 9:92–93
3. Kirtikar KR, Basu BD (1975) Indian medicinal plants, vol 2. Periodical Experts, Delhi, p 178
4. Nadkarni AK (1976) Indian materia medica, vol 1. Popular Prakashan, Bombay, p 468
5. Oudhia P 2003. Medicinal herbs of Chhattisgarh, India having less known uses of.XXXIV.Brahmadandi. http://www.botanical.com/site/column_poudhia/250_brahmadandi.html
6. Jain A, Katewa SS, Chaudhary BL, Galav P (2004) Folk herbal medicines used in birth control and sexual diseases by tribes of southern Rajasthan India. J Ethnopharmacol 90:171–177
7. Sharma KS, Mishra S, Mehta BK (1988) Antifertility activity of *Echinops echinatus* in albino rats. Ind J Med Sci 42:23–26
8. Chaturvedi M, Mali PC, Dixit VP (1995) Fertility regulation in male rats with the help of *Echinops echinatus* (Roxb) root extract. J Phytol Res 8:115–118
9. Anonymous (1996) Indian pharmacopoeia, vol 2. Government of India, New Delhi
10. Houghton PJ, Raman A (1998) Laboratory handbook for the fractionation of natural extracts. Chapman & Hall, London, p 118
11. Wagner H, Bladt S (1996) Plant drug analysis: a thin layer chromatography atlas, 2nd edn. Springer, Heidelberg, p 359
12. Gosh MN (1984) Fundamental of experimental pharmacology. Scientific Book Agency, Culcutta, pp 84–88
13. Bustos-Obregon E, Gonzalez-Hormazabal P (2003) Effect of a single dose of malathion on spermatogenesis in mice. Asian J Androl 5:105–107
14. Anonymous (1999) National committee for Clinical Laboratory Standards. Procedures for the handling and processing of blood specimens, 2nd edn. Approved guideline. NCCLS document M29–A42; Wayne (PA): NCCLS; October, p 40
15. Ulusoy E, Cayan S, Yilmaz N, Aktas S, Acar D, Doruk E (2004) Interferon α-2B may impair testicular histology including spermatogenesis in a rat model. Arch Androl 50:379–385
16. Gupta RS, Pramod-Kumar, Dixit VP, Dobhal MP (2000) Antifertility studies of the root extract of the *Barleria prionitis* Linn in male albino rats with special reference to testicular cell population dynamics. J Ethnopharmacol 70:111–117
17. Toney TW, Danzo BJ (1989) Estrogen and androgen regulation of protein synthesis by the immature rat epididymis. Endocrinology 125:231–242
18. Shan LX, Hardy MP (1992) Developmental changes in levels of luteinizing hormone receptor and androgen receptor in rat leydig cells. Endocrinology 131:1107–1114
19. Gupta G, Srivastava AK, Setty BS (1993) Androgenic regulation of glycolytic and HMP pathway in epididymides and vas deferens of rhesus monkey. Ind J Exp Biol 31:305–311
20. Lohiya NK, Ansari AS (1999) Male contraceptive agents. In: Joy KP, Krishna A, Haldar C (eds) Comparative endocrinology and reproduction. Narosa, New Delhi, pp 260–277
21. Topcu G (2006) Bioactive triterpenoids from *Salvia* species. J Nat Prod 69:482–487
22. Gupta RS, Bhatnager AK, Joshi YC, Sharma MC, Khushalani V, Kachhawa JBS (2005) Induction of antifertility with lupeol acetate in male albino rats. Pharmacology 75:57–62
23. Singh B, Ram SN, Pandey VB, Joshi VK, Gambhir SS (1991) Studies on anti-inflammatory activities of taraxasterol acetate from *Echinops echinatus* in rats and mice. Phytother Res 5:103–106