British Journal of Pharmacology (2003) 138, 117–120

www.nature.com/bjp

Restoration of chronic- Δ^9-THC-induced decline in sexuality in male rats by a novel benzoflavone moiety from *Passiflora incarnata* Linn

*,[1]Kamaldeep Dhawan & [1]Anupam Sharma

[1]Pharmacognosy Division, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh-160014, India

1 The present study comprised treatment of healthy male rats with Δ^9-tetrahydrocannabinol (THC, 10 mg kg^{-1}, p.o.), and combinations of THC with benzoflavone moiety (BZF, 10 and 20 mg kg^{-1}, p.o.) isolated from *Passiflora incarnata* Linneaus, over a period of 30 days.

2 Upon 30-days chronic administrations, the THC-treated male rats had a significant loss of libido (mounting behaviour with non-oestrous female rats), decrease in sperm count, and number of impregnated pro-estrous female rats.

3 Co-administration of BZF (10 and 20 mg kg^{-1} p.o.) afforded significant protection against the chronic-THC-induced decrease in libido, mating performance and fertility during 30-day experimental regimen. The 20 mg kg^{-1} dose of BZF exhibited better results.

4 Upon discontinuation of THC, treatment with BZF (10 and 20 mg kg^{-1} p.o.) also facilitated the early restoration chronic-THC-induced decline in libido, sperm count and sexual fertility within 7 days.

British Journal of Pharmacology (2003) **138,** 117–120. doi:10.1038/sj.bjp.0705015

Keywords: Aromatase; addiction; benzoflavone; cannabinoids; fertilization; libido; rats; sexuality

Abbreviations: BZF, tri-substituted benzoflavone; THC, delta-9-tetrahydrocannabinol

Introduction

Since time immemorial, the plant *Cannabis sativa* (family Moraceae) has been used by human beings for altering mood, thoughts, for the enhancement of sensual pleasures, and during religious occasions (Chopra, 1971). The resinous exudates from the dried flowering and fruiting tops of *Cannabis* contain over 61 cannabinoids, amongst which Δ^9-tetrahydrocannabinol (Δ^9-THC) is the main psychoactive phyto-moiety of *Cannabis* which accounts for all the biological effects of *Cannabis*. As per the latest report by the WHO, the abuse of cannabinoids (slang names like marihuana, marijuana, hash, pot, grass and hemp) over the last decade has become a very serious socio-economic health-care issue all-over the world (UNDCP, 2001). Besides addiction, chronic intake of cannabinoids has been reported to cause severe impairment of sexual functions in humans and animals (Russo *et al*., 2002). The decrease in libido, sperm count and fertility, induced by chronic *Cannabis* consumption in male species are some of the important issues which must be taken care of before prescribing cannabinoids in AIDS and cancer chemotherapy.

While working on a traditional plant *Passiflora incarnata* Linneaus (Passifloraceae, synonyms – passionflower, may-pops, marucuja, prempushpi), the methanol extract of the leaves of *P. incarnata* was confirmed to possess anxiolytic (Dhawan *et al*., 2001b, c), effects at a dose of 100 mg kg^{-1} *per oral* (p.o.) dose in mice. The bioactive methanol extract was further subjected to fractionations and chromatographic procedures to finally isolate a pure bioactive fraction (Dhawan, 2002). From this pure bioactive fraction, a tri-substituted benzoflavone moiety was isolated and its chemical identity was established using the standard spectroscopic techniques, i.e., UV, LC-MS, IR, ^{1}H-NMR and ^{13}C-NMR (Dhawan *et al*., 2001d, e; Dhawan, 2002). The isolation of a tri-substituted benzoflavone moiety (BZF) as the main bioactive phyto-constituent of *P. incarnata* has been an encouraging breakthrough in elucidating the mode of action of this plant, which finds mention in the ancient ayurvedic medical writings as a promising cure for male-impotence, post-menopausal decline in libido in females, menstrual irregularity, morphinism, alcoholism and tobacco addiction. Our subsequent pharmacological studies on the BZF moiety also confirmed that the BZF moiety isolated from *P. incarnata* was very effective in countering the menace of addiction-prone substances like morphine (Dhawan *et al*., 2002e), cannabinoids (Dhawan *et al*., 2002d), nicotine (Dhawan *et al*., 2002c) and ethanol (Dhawan *et al*., 2002f) in laboratory animals (Dhawan *et al*., 2002a). Treatments with 10 mg kg^{-1} p.o. dose of BZF for 30 days resulted to significant increase in the libido (mounting behaviour with non-estrous female rats,), sperm count, fertilization potential (number of pro-estrous female rats impregnated) and greater litter size, in 2-year-old male rats, relative to control group of animals which were given vehicle only (Dhawan *et al*., 2002b).

The encouraging results of our two recent studies on BZF showed (a) that BZF (10 and 20 mg kg^{-1}, p.o.) upon co-administration with Δ^9-THC (10 mg kg^{-1}, p.o.) twice daily, in a 6-day regimen, significantly attenuated the development of tolerance and dependence of cannabinoids in mice

*Author for correspondence; E-mail: kdd@glide.net.in

(Dhawan *et al*., 2002d), and (b) also increased the sperm count, libido, and sexual fertility during 30-day administration in 2-year-old male rats (Dhawan *et al*., 2002b), and prompted us to evaluate the BZF moiety for its probably usefulness in ameliorating or inhibiting the detrimental gonadal effects of chronic treatment with Δ^9-THC.

Methods

Plant material and the bioactive BZF moiety

Axial parts of cultivated *P. incarnata* at Rati Ram Nursery in the village of Khurammpur, Kalsia in the district of Saharanpur (UP, India) were picked in January 1999 and the identity was confirmed at the Department of Systematic Botany, Forest Research Institute, Dehradun (UP). A voucher specimen (code no. 1325/2000) was deposited in the Herbarium-cum-Museum of the Forest Research Institute, Dehradun, India. Studies were performed with the BZF moiety recently reported by us to have been isolated from this plant (Dhawan *et al*., 2001a, b, c, d, e; Dhawan, 2002).

Animals

Healthy adult male albino rats of Wistar strain (200–230 g) procured from the Disease Free Small Animals House, Haryana Agriculture University, Hisar (Haryana) were bred at the Central Animal House of the Panjab University, Chandigarh. The experimental protocols were approved by the Institutional Ethical Committee of the Panjab University, Chandigarh. The animals were divided as:

(1) Control group – given vehicle (sesame oil containing 1% w w^{-1} Polysorbate 80)
(2) THC group – given 10 mg kg^{-1} Δ^9-THC in vehicle
(3) THC + BZF-10 group – given THC (10 mg kg^{-1}) concurrently with BZF (10 mg kg^{-1})
(4) THC + BZF-20 group – given THC (10kg^{-1} concurrently with BZF (20 mg kg^{-1})
(5) BZF-10 group – given 10 mg kg^{-1} BZF moiety
(6) BZF-20 group – given 20 mg kg^{-1} BZF moiety

All the treatments were administered *per oral* (p.o.) once a day for 30 days. Each treated group comprised eight male rats ($n=8$; however in THC group $n=12$). At the end of treatments, the treated male rats were paired with non-oestrous female rats for 15 min and the number of mounts was recorded to assess the male rats libido. On the 31st day the male rats were mated with fertile pro-oestrous female rats (1 : 3) in separate cages for each different group. The presence of spermatozoa in the vaginal plug was taken as a parameter for mating. The number of female rats who became pregnant, was considered to be a parameter of fertility.

Mounting behaviour at the end of treatments on day 30

To observe the libido-oriented mounting behaviour, non-oestrous female rats were paired with treated male rats. The male rat assuming the copulatory position over the female but failing to achieve intromission was considered as a mount (Subramoniam *et al*., 1997). Male rats from each group were randomly chosen and suitably marked. The rats were placed in a clear aquarium and allowed to acclimatize for 15 min. After that, a non-oestrous female was introduced into the arena. The number of mounts was recorded for 15 min.

Sperm count and fertilization determined on day 31

The spermatozoa were counted as per the method of Zaneveld & Polakoski (1997). Sperm suspension was placed on both sides of Neubauer's hemocytometer and allowed to settle for 1 h. The number of spermatozoa in the squares of the hemocytometer was counted under the microscope at $100\times$ magnification. The relative sperm count after different treatments, and the female rats impregnated by male rats (3 : 1) after receiving various treatments were counted.

Determination of sexual behaviour upon BZF administration to THC-treated rats

After evaluating their sexual behaviour and sexual parameters, the 12 male rats of THC groups were further subdivided into control, BZF-10 and BZF-20 groups. The control group animals were given vehicle, whereas the other animals were given 10 and 20 mg kg^{-1} BZF, p.o., once a day. The rationale for doing this study was to determine the beneficial effects of BZF-administration in restoring sexual parameters after cessation of chronic treatment with cannabinoids. On the 7th day after cessation of these treatments the sexual behaviour and parameters were again evaluated.

Results

As shown in the Table 1, rats subjected to 30-days chronic THC-treatment incurred loss of libido which was evident from their mounting behaviour pattern. Chronic administration of THC to male rats also rendered them sterile (sperm count $=72.8\times10^6$), and these rats failed to impregnate any female rat. However, the concurrent administrations of THC with BZF for 30 days, afforded a significant protection of the loss of libido and sexual parameters in rats receiving these co-treatments. Male rats receiving 10 mg kg^{-1} p.o. dose of BZF + THC for 30 days exhibited elevated sperm count, i.e., 381.1×10^6, and impregnated 62.5% of the female rats with reference to the THC group. The male rats receiving THC and 20 mg kg^{-1} dose of BZF exhibited still better results (sperm count $=418.0\times10^6$; and female rats impregnated $=87.5\%$). Administrations of BZF (10 and 20 mg kg^{-1}, p.o.) for 30 days in male rats increased libido, sperm count and number of female rats impregnated (Last two rows of Table 1).

Sexual parameters, with and without the co-administration of BZF (10 and 20 mg kg^{-1} BZF) in chronic cannabinoid-treated rats were evaluated on day 7 (Table 2). Upon measuring the various sexual parameters on day 7, the male rats receiving BZF-10 exhibited improvement in the sexual parameters. However mice which were given BZF-20 exhibited very significant recovery (almost 100%) in sexual performance, libido, and sperm count, relative to the rats of the control group (these are originally the male rats who received THC for 30 days, as indicated in Table 1, and were

Table 1 Mounting behaviour, sperm count, and mating performance after treatments for 30 days

Parameter	*Control*	*THC*	*THC + BZF-10*	*THC + BZF-20*	*BZF-10*	*BZF-20*
Mounting behaviour of treated male rats recorded for 15 min on day 30	8.4 ± 1.95	0.0	6.7 ± 0.94*	12.5 ± 1.64**	18.8 ± 1.47**	22.8 ± 1.87***
Sperm count (10^6) of treated male rats after recorded treatments for 30-days	412.3 ± 11.99	72.8 ± 4.13***	381.1 ± 12.9**	418.0 ± 7.92	444.3 ± 8.23**	455.0 ± 11.08***
Females impregnated by male rats	15 (62.5%)	0	15 (62.5%)	21 (87.5%)	24 (100%)	24 (100%)

Sperm count and number ofmounts is expressed as mean ± s.d.mean. $n = 8$ (male rats in various groups, except THC group where $n = 12$), $n = 24$ (female rats); $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ versus Control. ANOVA followed by Fischer's LSD test. Non-oestrous female rats used for evaluating mounting behaviour; Pro-oestrous female rats used for evaluation of serual fertilization. THC (Δ^9-tetahydrocannabinol), BZF (benzoflavone moiety of *P. incarnata*), BZF-10 (10 mg kg^{-1} dose of BZF), BZF-20 (20 mg kg^{-1} dose of BZF)

Table 2 Sexual behaviour and parameters recorded on day 7 after treatment of THC group with BZF

Parameter	*Control*	*BZF-10*	*BZF-20*
Mounting behaviour of treated male rats recorded for 15 min on day 30	0	10.4 ± 0.57***	15.7 ± 1.21***
Sperm count (10^6) of treated male rats after recorded treatments for 30-days	82.0 ± 6.08	398.0 ± 6.87***	410.7 ± 10.69***
Females impregnated by male rats	0	11 (91.7%)	12 (100%)

Sperm count and number ofmounts is expressed as mean ± s.d.mean. $n = 4$ (number of male rats) $n = 12$ (female rats); $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ versus Control. ANOVA followed by Fischer's LSD test. Non-oestrus female rats used for evaluating mounting behaviour; Pro-oestrus female rats used for evaluation of secual fertilization. THC (Δ^9-tetahydrocannabinol), BZF (benzoflavone moiety of *P. incarnata*), BZF-10 (10 mg kg^{-1} dose of BZF), BZF-20 (20 mg kg^{-1} dose of BZF)

again treated with vehicle only for the next 7 days) and were not given BZF at all during the entire experimental paradigm.

Discussion

It is evident from the findings reported in Table 1 and 2 that the co-treatment of BZF + THC, significantly prevents the deleterious sexual-effects of chronic THC treatments in male rats. In addition to this, BZF also facilitates the early restoration of sexual parameters after cessation of chronic treatment with THC. In both studies, the 20 mg kg^{-1} dose of BZF affords better results than the BZF-10 dose. The rats, which received only BZF (10 and 20 mg kg^{-1}), exhibited increased libido, increased sperm count, and had increased virility.

Chronic intake of cannabinoids (Block *et al*., 1991; Rosenkrantz & Esber, 1980) leads to severe detrimental effects on the sex hormones production (by increasing oestrogen levels). The increase in free oestrogens in males species causes loss of libido, enlarged prostrate, fatigue and loss of muscle tone. Further, the increased oestrogen levels cause the disruption of normal testicular production of testosterone, and saturation of testosterone receptors in the hypothalamus in brain, thereby reducing the signal sent to the pituitary gland which in turn reduces the secretion of LH (Kaliszuk *et al*., 1989). This ultimately reduces testosterone production in gonads. Increased oestrogen due to excessive cannabinoid-intake also increases the production of sex-hormone binding globulin (SHBG) (Sharpe, 1998). The SHBG binds testosterone and reduces the free testosterone in the blood. The mode of action of the BZF moiety is an interesting and simple neuro-steroidal phenomenon. The BZF moiety, reported for the first time from a plant source, is a very potent inhibitor (Kao *et al*., 1998; Kellis & Vickery, 1984) of the liver microsomal enzyme aromatase, which is a member of cytochrome P-450 family (Lee *et al*., 1994b). The aromatase (P-450 3A4) enzyme is accountable for the metabolism of more than half of the drugs in use today (Guengerich, 1999), including the sex steroids, e.g., testosterone (Lee *et al*., 1994a). A specific functional aromatase gene 'CYP 19' is necessary for the expression of aromatase enzyme in mice and human beings (Robertson *et al*., 1999). The enzyme aromatase is instrumental in the metabolism of testosterone to estradiol (Wilson, 1995). The BZF moiety, the strongest aromatase-inhibitor, prevents the metabolic de-activation of testosterone to estradiol (Wilson, 1995). The BZF moiety, the strongest aromatase-inhibitor, prevents the metabolic de-activation of testosterone to estradiol and, thus, increases free testosterone and decreases free oestrogen (Chen *et al*., 1997; Merken & Beecher, 2001). The increased plasma-testosterone levels directly influence the gonadotropins (luteinizing *LH* and follicle-stimulating-hormone *FSH*), which regulate spermatogenesis and maturation of sperms (Crémoux, 2000). High levels of testosterone in male species mean sexuality, increased libido, mental energy, stamina and virility.

The BZF moiety isolated from aerial parts of *P. incarnata* acts: (a) through its anti-aromatase function, and (b) by eliminating oestrogen's negative feedback loop (Chen *et al*., 1997). The concomitant administration of BZF with THC is postulated to maintain a high level of testosterone in the blood by stopping the aromatization of testosterone to oestrogen. Secondly, BZF may also facilitate the body to produce more testosterone by eliminating the

so-called 'negative feedback' loop, that otherwise reduces natural testosterone production. High testosterone accounts for an increase in sperm count, fertility, as well as sexual drive. Thus, from these studies the authors feel encouraged that the novel BZF moiety, being a highly potent anti-oxidant and the strongest reported aromatase inhibitor, affords significant prevention against the deleterious effects of substances like cannabinoids upon male sexuality, vigour and fertility.

The authors are grateful to the Council of Scientific and Industrial Research, New Delhi, India for providing financial support through the award of Senior Research Fellowship to Kamaldeep Dhawan.

References

BLOCK, R.I., FARINPOUR, R. & SCHLECHTE, J.A. (1991). Effects of chronic marijuana use on testosterone, luteinizing hormone, follicle stimulating hormone, prolactin and cortisol in men and woman. *Drug Alc. Depend.,* **28,** 121 – 128.

CHEN, S., KAO, Y.C. & LAUGHTON, C.A. (1997). Binding characteristics of aromatase inhibitors and phytoestrogens to human aromatase. *J. Steroid Biochem. Mol. Biol.,* **61,** 107 – 115.

CHOPRA, I.C. (1971). Drug Addiction. *Indian J. Pharmacol.,* **3,** 43 – 50.

CRÉMOUX, P. (2000). Aromatase inhibitors: pharmacological aspects. *Bulletin du Cancer,* **87,** 23 – 30.

DHAWAN, K. (2002). Phytochemical, Biological and Standardization Studies on *Passiflora incarnata* Linn. Chandigarh-160014, India: Thesis submitted for the award of Doctor of Philosopy (Pharmaceutical Sciences) of the Panjab University.

DHAWAN, K., KUMAR, R., KUMAR, S. & SHARMA, A. (2001a). Correct Identity of *Passiflora incarnata* Linneaus: A Promising Anxiolytic and Sedative. *J. Med. Food,* **3,** 143 – 149.

DHAWAN, K., KUMAR, S. & SHARMA, A. (2001b). Comparative biological activity study on *Passiflora incarnata* and *P. edulis*. *Fitoterapia,* **72,** 698 – 702.

DHAWAN, K., KUMAR, S. & SHARMA, A. (2001c). Anxiolytic activity of aerial and underground parts of *Passiflora incarnata*. *Fitoterapia,* **72,** 922 – 926.

DHAWAN, K., KUMAR, S. & SHARMA, A. (2001d). Anti-anxiety studies on extracts of *Passiflora incarnata* Linneaus. *J. Ethnopharmacol.,* **78,** 165 – 170.

DHAWAN, K., KUMAR, S. & SHARMA, A. (2001e). *Passiflora incarnata* Linn: A Promising Anxiolytic and Sedative - Isolation of a benzoflavone moiety as the bioactive agent. Presentation at the 42nd Annual Meeting of the American Society of Pharmacognosy, July 14 – 18, 2001, at Mexico, published in the Abstract Book as Oral Presentation - 29.

DHAWAN, K., KUMAR, S. & SHARMA, A. (2002a). Attenuation of drug/substance dependence by a novel tri-substituted benzoflavone moiety from *Passiflora incarnata* Linneaus. New Brunswick, New Jersey: Presentation at the 43rd Annual Meeting of the American Society of Pharmacognosy. Published in the Abstract Book at O-16.

DHAWAN, K., KUMAR, S. & SHARMA, A. (2002b). Beneficial Effects of chrysin and Benzoflavone on Virility in 2-Year-Old Male Rats. *J. Med. Food,* **5,** 43 – 47.

DHAWAN, K., KUMAR, S. & SHARMA, A. (2002c). Nicotine reversal effects of the benzoflavone moiety from *Passiflora incarnata* Linneaus in mice. *Addiction Biol.,* **7,** 435 – 441.

DHAWAN, K., KUMAR, S. & SHARMA, A. (2002d). Reversal of cannabinoids (Δ^9-THC) by the benzoflavone moiety from methanol extract of *Passiflora incarnata* Linneaus in mice: a possible therapy for marihuana addiction. *J. Pharm. Pharmacol.,* **54,** 875 – 881.

DHAWAN, K., KUMAR, S. & SHARMA, A. (2002e). Reversal of tolerance and dependence of morphine by *Passiflora incarnata* Linneaus - A traditional medicine to combat morphine addition. *Pharma. Biol.,* **40,** (in press).

DHAWAN, K., KUMAR, S. & SHARMA, A. (2002f). Suppression of alcohol-cessation-oriented hyper-anxiety by the benzoflavone moiety of *Passiflora incarnata* Linneaus in mice. *J. Ethnopharmacol.,* **81,** 239 – 244.

GUENGERICH, F.P. (1999). Cytochrome P-450 3A4: Regulation and role in drug metabolism. *Annu. Rev. Pharmacol. Toxicol.,* **39,** 1 – 17.

KALISZUK, S., BORZECKI, Z. & SWIES, Z. (1989). The influence of bromocriptine on sexual activity in ethanol-exposed male rarts. *Annales Universitatis Mariae Curie-Sklodowska. Sectio D, Medicina,* **44,** 109 – 114.

KAO, Y.C., ZHOU, C., SHERMAN, M., LAUGHTON, C.A. & CHEN, S. (1998). Molecular basis of the inhibition of human aromatase (estrogen synthetase) by falvone and isoflavone phytoestrogens: A site-directed mutagenesis study. *Environment. Health Persp.,* **106,** 85 – 92.

KELLIS, J.T. & VICKERY, L.E. (1984). Inhibition of estrogen synthetase (aromatase) by flavones. *Science,* **225,** 1032 – 1033.

LEE, H.S., JIN, C., PARK, J. & KIM, D.H. (1994a). Involvement of P4503A in the metabolism of 7,8-benzoflavone by human liver microsomes. *Xenobiotica,* **24,** 1053 – 1062.

LEE, H.S., JIN, C., PARK, J. & KIM, D.H. (1994b). Modulation of cytochrome P450 activities by 7,8-benzoflavone and its metabolites. *Biochem. Mol. Biol. Int.,* **34,** 483 – 491.

MERKEN, H.M. & BEECHER, G.R. (2001). *Finessing the Flavonoids: A part of Human Nutrituion*, an ARS National Program. Beltsville, MD: U.S. Government Printing Office.

ROBERTSON, K.M., DONNELL, L.O., JONES, M.E., MEACHAM, S.J., BOON, W.C., FISHER, C.R., GRAVES, K.H., MCLACHLAN, R.I. & SIMPSON, E.R. (1999). Impairment of spermatogenesis in mice lacking a functional aromatase (*cyp* 19) gene. *Develop. Biol.,* **96,** 7986 – 7991.

ROSENKRANTZ, H. & ESBER, H.J. (1980). Cannabinoid-induced hormone changes in monkeys and rats. *J. Toxicol. Environ. Health,* **6,** 297 – 313.

RUSSO, E., MATHRE, M.L., BYRNE, A., VELIN, R., BACH, P.J., RAMOS, J.S. & KIRLIN, K.A., (2002). Chronic cannabis use in the compassionate investigational new drug program: an examination of benefits and adverse effects of legal clinical cannabis. *J. Cannabis Therapeutics,* **2,** 3 – 58.

SHARPE, R.M. (1998). Natural and antropogenic environmental oestrogens: the scientific basis for risk assessment – Environmental oestrogens and male infertility. *Pure & Appliked Chemistry,* **70,** 1685 – 1701.

SUBRAMONIAM, A., MADHAVACHANDRAN, R., RAJASEKHARAN, S. & PUSHPANGADAN, P. (1997). Aphrodisiac property of *Trichopus zeylancus* extract in male mice. *J. Ethnopharmacol.,* **57,** 21 – 27.

UNDCP (2001). Global Illicit Drug Terends 2001 – Reduction of illicit demand for drugs: world situation with regard to drug abuse. Vienna: Report issued by the United Nationals Office for Drug Control and Crime Prevention: Commission on Narcotic Drugs, 43rd session.

WILSON, J.D. (1995). Androgens. In: Goodman and Gilman's *The Pharmacological basis of therapeutics*. 9th edn. pp 1441 – 1457. New York: McGraw-Hill.

ZANEVELD, L.J.D. & POLAKOSKI, K.L. (eds) (1997). Collection and physical examination of the ejaculate. In: *Techniques of Human Andrology*. pp 147 – 156. Amsterdam: North Holland Biomedical Press.

(Received May 30, 2002
Revised September 6, 2002
Accepted October 1, 2002)