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Reversal of cannabinoids (Δ^9-THC) by the benzoflavone moiety from methanol extract of *Passiflora incarnata* Linneaus in mice: a possible therapy for cannabinoid addiction

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Abstract

The newly reported benzoflavone moiety from the plant *Passiflora incarnata* Linneaus has been evaluated in light of traditional reports on the use of *P. incarnata* in breaking down cannabis addiction. In the modern or allopathic system of therapeutics, there has been no suitable remedy to combat the severe withdrawal effects of various cannabis products, including marihuana, marijuana, bhang, hashish, ganja, etc., the world-wide consumption of which has attained alarming proportions especially among the younger generation. Mice were given a 10-mg-kg^{-1} twice-daily dose of Δ^9-tetrahydrocannabinol (Δ^9-THC) by mouth for six days to make them dependent upon cannabinoids. Concurrently, other groups of mice were administered Δ^9-THC along with a 10- or 20-mg-kg^{-1} twice-daily dose of the benzoflavone moiety from *P. incarnata* orally for 6 days. Upon measuring locomotor activity during the treatment regimen, it was noticed that the mice receiving the *P. incarnata* extract and Δ^9-THC together developed significantly less tolerance and dependence, relative to the mice receiving Δ^9-THC alone. Upon administration of SR-141716A, a selective cannabinoid-receptor antagonist (10 mg kg^{-1}, p.o.) to all the groups of mice on the 7th day, an artificial withdrawal was produced due to an abrupt decline of Δ^9-THC levels in mouse brain. However, the typical withdrawal effects like paw tremors and head shakes were significantly less in the mice given Δ^9-THC+*P. incarnata* benzoflavone moiety for 6 days. Upon administration of 20 mg kg^{-1} of the *P. incarnata* benzoflavone moiety to mice showing severe symptoms of withdrawal due to administration of SR-141716A, there was a marked attenuation of withdrawal effects, thereby suggesting the usefulness of the benzoflavone moiety in Δ^9-THC withdrawal. Thus, the benzoflavone moiety of *P. incarnata*, when administered concurrently with Δ^9-THC, prevented the development of tolerance and dependence of cannabinoids in mice. Even an acute administration of the benzoflavone moiety (20 mg kg^{-1}, p.o.) significantly blocked the expression of withdrawal effects in Δ^9-THC-dependent mice.

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Introduction

For many hundreds of years, the plant *Cannabis sativa* (Moraceae) has been used for altering mood and thoughts, for the enhancement of sensual pleasures, and during religious occasions (Chopra 1971). Ancient Hindu, Chinese and Arabic medical literature also mentions *C. sativa* (bhang, ganja or hashish) for its hypnotic, anticonvulsant, anxiolytic, appetite-stimulant and anti-tussive properties. During Napoleon's Egyptian expedition, the plant attracted the Europeans who further

spread it to all parts of the world (Evans 2001). The resinous exudates from the dried flowering and fruiting tops of cannabis contain over 61 cannabinoids of which Δ^9-tetrahydrocannabinol (Δ^9-THC) has been reported to be the main psychoactive phyto-moiety (INFOFAX 2001). In a naïve user cannabinoids produce mild euphoria, joviality and enhanced pleasure obtained from any work of art. Tolerance to most of the effects of cannabinoids (or, practically, Δ^9-THC) develops after a few doses, and symptoms of physical and psychological dependence comprise antinociception, hypothermia, depression of locomotor activity, catalepsy, ataxia, anticonvulsant activity and hypotension. Deprival of cannabinoids in a chronic user causes appearance of severe withdrawal effects in the form of restlessness, hyperirritability, insomnia, aggression, tremors, twitching, nausea and sleep-electroencephalogram disturbances (Haney et al 1999). The medicinal use of cannabinoids in cancer chemotherapy and AIDS has become a debatable concern in light of their addiction liabilities. Cannabinoid addicts are highly susceptible to premature memory impairment, have unpredictable social relationships and usually suffer from amotivational syndrome due to lack of interest, activity, drive and motivation in life (O'Brien 1996). A recent report by the World Health Organization (WHO) provides incontrovertible evidence that the abuse of cannabinoids over the last decade has become a very serious global health-care issue (Connell et al 2001; INFOFAX 2001; Julin 2001; Maca 2001; Nadelmann 2001; Stoke 2001; Thompson 2001; UNDCP 2001; Walluks 2001). In the USA, over 72 million adult citizens have resorted to consumption of cannabinoids at least once in their life time. The life-time consumption of cannabinoids (Δ^9-THC, marihuana, marijuana, hash, boom, etc.) by adult populations in some other countries has been reported as: Australia (44%), Canada (28%), UK (14%), Sweden (8%), Finland (5%), Brazil (5%), Colombia (5%), Ecuador (4%), India (3%), and the Dominican Republic (1%). Seizures of illicit cannabis have been made from India, Jordan, Lebanon, Pakistan, Thailand, Nepal and Laos. Although consumer data are scarce in Africa, prevalence of this problem has been confirmed from the reports of seizures of cannabis from Algeria, Kenya, Lesotho, Malawi, Morocco, Nigeria, Senegal and South Africa. Data generated by the National Institute of Drug Abuse during 1999 have revealed that over 11.2% of 6^{th}-, 8^{th}- and 10^{th}-grade students consumed marijuana daily in the USA.

Except for a few antidepressant drugs, no specific treatment is available in the modern system of therapeutics to deal with cannabinoid addiction and dependence (O'Brien 1996). The tendency of a chronic user seeking refuge in marijuana again always persists, as the residual effects of Δ^9-THC last for several weeks in the body due to its deposition in body fats. In the traditional system of medicine in India, *Passiflora incarnata* Linneaus (Passifloraceae; synonyms – passionflower, maracuja, maypops, prempushpi) attracted our attention through various reports of its use in breaking down cannabis habit in addicts (Vasudev 1955; Brounstein 1995 (personal communication); Johnson 1995; Thorpe 2001). *P. incarnata* has been used in all parts of the world as a plant-derived anxiolytic and sedative, and forms an active constituent of as many as 130 herbal, homoeopathic and allopathic medicinal preparations used for the treatment of various cardiovascular, respiratory and nervous-system disorders (Reynolds 1996). We reported recently that a methanol extract of aerial parts of *P. incarnata* exhibits significant anxiolytic effect at a 125-mg-kg^{-1} oral dose in mice, using the elevated plus-maze model of anxiety (Dhawan et al 2001a). Leaves of *P. incarnata* had the greatest anxiolytic effects, whereas the roots were devoid of any activity (Dhawan et al 2001b). Methanolic extract of leaves of *P. incarnata* exhibited good anti-tussive properties against SO_2-induced cough in mice (Dhawan & Sharma 2001) and also had aphrodisiac properties when 100 mg kg^{-1} was given by mouth to male mice (Dhawan et al 2001c). The same dose prevented acetylcholine-induced bronchospasm in guinea-pigs, thus inferring that the methanol extract of leaves of *P. incarnata* had anti-asthmatic and spasmolytic properties (Dhawan et al 2001d).

Resorting to bioactivity-directed fractionation and chromatographic procedures, we have been able to isolate and identify a new bioactive benzoflavone phyto-moiety from the methanol extract of aerial parts of *P. incarnata* (Dhawan et al 2001e, f, g). This bioactive benzoflavone compound has been confirmed as being liable for the multifarious biological effects of *P. incarnata* in our subsequent studies. The anxiolytic effect produced by 10 mg kg^{-1} of the benzoflavone moiety were significantly better than that exhibited by diazepam (2 mg kg^{-1}, p.o.) in mice (Dhawan et al 2001e). The benzoflavone moiety of *P. incarnata* has shown encouraging results in treating morphine addiction by reversing the tolerance and dependence produced by chronic, as well as acute, treatment of mice with morphine (Dhawan et al 2001h, i).

In this study, we have made an attempt to evaluate the possible usefulness of the benzoflavone moiety of *P. incarnata* in mice, by rendering the mice dependent on Δ^9-THC. We employed a dose regimen that would

produce pharmacological and behavioural tolerance. Mice were divided into four major groups: control group, given vehicle only; THC group, given Δ^9-THC; THC + benzoflavone group, given Δ^9-THC and different doses of benzoflavone; and benzoflavone group, given different doses of benzoflavone. All the treatments were administered twice daily for 6 days. Twenty-four hours after the cessation of treatments on the 6th day, each group was given SR-141716A (a potent and selective antagonist of brain cannabinoid receptors) (Cook et al 1998) and the behavioural pattern of each group was recorded. Additionally, the locomotor activity of all the groups was recorded on the 5th day, and also after the administration of the cannabinoid-receptor antagonist on the 7th day.

Materials and Methods

Plant material

Aerial parts of *P. incarnata* were picked in January 1999 from a cultivated source at Rati Ram Nursery, village Khurammpur via Kalsia, district Saharanpur (UP, India). The identity of the procured plant material was confirmed by the Department of Systematic Botany, Forest Research Institute, Dehradun (UP, India). A voucher specimen (code no. 1325/2000) was deposited in the Herbarium-cum-Museum of the Forest Research Institute, Dehradun.

Extraction, fractionation and isolation of the bioactive benzoflavone

The aerial parts were dried in shade and powdered (no. 60) and 100 g of the dried powder was Soxhlet-extracted successively with petroleum ether (60–80°C), chloroform (Ranbaxy Laboratory Chemicals), methanol (sd Fine-Chem Limited) and distilled water. All the extracts were dried using a Buchi 461 Rotary Vacuum Evaporator and were preserved in a vacuum desiccator containing anhydrous silica blue. The weight of the extracts after drying was calculated as: petroleum ether extract, 6.8875 g; chloroform extract, 8.2314 g; methanol extract, 11.8787 g; and water extract, 4.8876 g. The four different extracts of *P. incarnata* were suspended in a vehicle comprising Simple Syrup I.P. and 1% w/w carboxymethylcellulose as suspending agent. Five sets of doses (300, 200, 125, 100 and 75 mg kg^{-1}) of each *P. incarnata* extract were prepared by suspending the dried extracts in the vehicle under vigourous stirring to form a uniform suspension. The weight of the dried extracts was so adjusted as to administer 0.25 mL of the suspension. Simple syrup containing carboxymethylcellulose (0.25 mL) was used as control. Of the four extracts, only the methanol extract of *P. incarnata* showed a significant anxiolytic activity at a dose of 125 mg kg^{-1} whereas the remaining three extracts did not exhibit anxiolytic activity statistically comparable with that of the standard anxiolytic (diazepam, 2 mg kg^{-1} in vehicle, p.o.). The bioactive methanol extract was processed and purified further by resorting to bioactivity-directed fractionation and chromatographic procedures until a fraction which exhibited significant anxiolytic activity at a dose of 10 mg kg^{-1} in mice was obtained. This fraction (yield = 332 mg, 0.33%) tested positive for the presence of flavones. Thin-layer chromatography of the final bioactive fraction exhibited a blue fluorescent spot under UV light (366 nm) at R_f 0.65 using the mobile phase petroleum ether–toluene–ethyl acetate–acetone (13:4:2:1). UV, LC-MS, GC-MS, IR, ^{1}H NMR, and ^{13}C NMR characterisation studies have confirmed the presence of a benzoflavone moiety from *P. incarnata*, never previously reported, that accounts for the central nervous system properties of *P. incarnata* (Dhawan et al 2001d, e, f, i). This compound has a basic structure comprising a benzene ring fused at the 6,7 position of a flavone compound. The exact structure and chemical identity of the benzoflavone moiety is not being presented here due to patent considerations.

In this study, the Δ^9-THC reversal effects of *P. incarnata* were examined at two different doses of benzoflavone, 10 and 20 mg kg^{-1}.

Animals

Swiss albino mice (either sex) procured from the Disease Free Small Animals House, College of Veterinary Sciences, Haryana Agriculture University, Hisar, India, were bred at the Central Animal House of the Panjab University, Chandigarh. The mice were allowed free access to standard laboratory feed and water. Groups of ten mice (22–24 g) were used in all sets of experiments. Tolerance and dependence to Δ^9-THC was induced in mice by administration of Δ^9-THC (10 mg kg^{-1}, p.o.) twice daily at 0900 and 1700 h for 6 days. The experimental protocols were approved by the institutional Small Animals Ethical Committee.

Drugs

Δ^9-Tetrahydrocannabinol (Δ^9-THC) and its specific antagonist SR-141716A were provided as gift samples by Prof. M. L. Chhabra, Scientist, SOEL, Haryana Agriculture University, Hisar, Haryana, India. All the treat-

ments were administered through a vehicle comprising sesame oil containing 1% w/w Polysorbate 80. The volume of single administration was 0.25 mL. The mice were deprived of food for 18 h before the experiment and allowed water only.

Treatment regimen

Each group of 10 mice were given the following treatments orally: control group, 0.25 mL vehicle, twice daily for 6 days; THC group, 10 mg kg^{-1} Δ^9-THC in vehicle, twice daily for 6 days; THC+benzoflavone groups, 10 mg kg^{-1} Δ^9-THC in vehicle+10 or 20 mg kg^{-1} benzoflavone in vehicle, twice daily for 6 days; benzoflavone groups, 10 or 20 mg kg^{-1} of benzoflavone in vehicle, twice daily for 6 days. This 6-day regimen was reported to produce tolerance to various pharmacological effects of Δ^9-THC, including locomotor activity and anxiogenic behaviour (Vogel & Vogel 1997; Cook et al 1998).

Locomotor activity

The locomotor (ambulatory) activity of mice was measured using a computerized Animal Activity Meter (Opto Varimex Mini, Columbus Instruments, OH). An array of 15 infra-red emitter/detector pairs, spaced at 2.5-cm intervals, measured the animal activity along a single axis of motion, the digital data being displayed on the front panel meters as ambulatory activity. The mice were placed individually in a transparent plastic cage (29 cm × 22 cm × 22 cm) for 5 min and the ambulatory activity was recorded.

Tolerance development and administration of the specific antagonist

After the twice daily administration of the various treatments for 6 days, 20 mg kg^{-1} of SR-141716A was administered orally to all the groups at 0900 h on the 7th day and all the groups were monitored very carefully for a span of 30 min. The unique behaviour of abrupt withdrawal/cessation of Δ^9-THC comprised head shakes, paw tremors, writhing, scratching, grooming, piloerection and straub tail. Paw tremors were rapid lateral movements of the paws that could be counted. The grooming episode comprised licking of paws and body, and rubbing paws over nose, head, ears. These parameters were recorded for all the groups.

Results and Discussion

The ambulatory activity counts in mice recorded on the 5th day (i.e. during the treatment regimen) are shown in Table 1. The locomotor activity counts for the different groups of treated mice after the administration of SR-141716A on the 7th day are recorded in Table 2. The head shakes and paw tremors recorded after the administration of SR-141716A in the different groups of mice are presented in Table 3.

The mice in the THC group were segregated into two sub-groups of 5 mice each and were labelled as THC-A and THC-B groups. Mice in the THC-B group were given 20 mg kg^{-1} of benzoflavone as a single oral dose and were separated from the untreated mice. One hour after the acute administration of benzoflavone to the 5 mice in group THC-B, the severity of paw tremor and head shakes was again measured. The recordings are presented in Table 4.

It is evident from Table 1 that administration of Δ^9-THC twice daily for 5 days induced its pharmacological effects, as seen from the decreased locomotor activity in mice which received 10 mg kg^{-1} of Δ^9-THC twice daily. There was a marked decrease in locomotor activity, indicative of the lethargic attitude and lack of drive in the THC-intoxicated mice. Interestingly, the groups of mice concurrently receiving two doses of the benzoflavone moiety of *P. incarnata* exhibited greater locomotor activity relative to the control as well as to the THC-intoxicated mice. These findings, though preliminary, suggest the beneficial effects of the bioactive phyto-moiety of *P. incarnata*. The increased locomotor behaviour in mice receiving Δ^9-THC+benzoflavone concurrently, recorded on the 5th day, suggests the usefulness of the benzoflavone moiety in delaying the development of tolerance to Δ^9-THC in mice in a dose-dependent manner. The 20-mg-kg^{-1} oral dose of the benzoflavone moiety showed better results.

When recorded after the administration of SR-141716A, there was a marked increase in the locomotor activity exhibited by mice in the THC group relative to the control group, and also relative to the locomotor activity of the same mice recorded on the 5th day. However, it is evident from Table 2 that the mice groups given THC+benzoflavone did not exhibit much difference in ambulatory behaviour even after the administration of the antagonist SR-141716A when a comparison of their locomotor activity with the corresponding value recorded on the 5th day was made. A close perusal of the ambulatory behaviour of the mice receiving THC+benzoflavone together reveals that their ambulatory behaviour was not affected much by the administration of SR-141716A (the 20-mg-kg^{-1} dose of the benzoflavone moiety rendering the behaviour even more similar). Administration of a specific cannabinoid receptor antagonist like that of SR-

Table 1 Locomotor activity recorded in mice on the 5th day of a treatment regimen of 6 days.

Treatment	Ambulatory activity counts
Control (vehicle 0.25 mL)	121.3±11.3
THC (10 mg kg^{-1})	105.6±5.8[a]
THC+*P. incarnata* benzoflavone moiety (10 mg kg^{-1})	172.7±12.0**
THC+*P. incarnata* benzoflavone moiety (20 mg kg^{-1})	198.6±11.2*
P. incarnata benzoflavone moiety (10 mg kg^{-1})	208.0±13.0
P. incarnata benzoflavone moiety (20 mg kg^{-1})	212.5±14.2*

Data are expressed as mean±s.d. (n = 10). THC = Δ^9-tetrahydrocannabinol. Doses were given twice daily, by mouth, for 6 days *$P < 0.05$, **$P < 0.01$ with respect to benzoflavone moiety (10 mg kg^{-1}); [a]$P < 0.05$ vs control; analysis of variance followed by Fischer's LSD test.

Table 2 Locomotor activity in mice after administration of the Δ^9-tetrahydrocannabinol antagonist SR-141716A on 7th day.

Treatment	Ambulatory activity counts
Control (vehicle 0.25 mL)	117.3±11.0
THC (10 mg kg^{-1})	136.2±11.4[a]
THC+*P. incarnata* benzoflavone moiety (10 mg kg^{-1})	181.3±8.7**
THC+*P. incarnata* benzoflavone moiety (20 mg kg^{-1})	203.8±6.6
P. incarnata benzoflavone moiety (10 mg kg^{-1})	205.8±12.2
P. incarnata benzoflavone moiety (20 mg kg^{-1})	209.5±8.8*

Data are expressed as mean±s.d. (n = 10). THC = Δ^9-tetrahydrocannabinol. Doses of THC and the benzoflavone moiety were given twice daily, by mouth, for 6 days; SR-141716A 20 mg kg^{-1} was given by mouth on day 7. *$P < 0.05$, **$P < 0.01$ with respect to benzoflavone moiety (10 mg kg^{-1}); [a]$P < 0.05$ vs control; analysis of variance followed by Fischer's LSD test.

Table 3 Paw tremors and head shakes recorded for 15 min in mice 1 h after administration of SR-141716A.

Treatment	Paw tremors	Head shakes
Control (vehicle 0.25 mL)	–	–
THC (10 mg kg^{-1})	129.0±5.36	24.3±3.2
THC+*P. incarnata* benzoflavone moiety (10 mg kg^{-1})	71.4±3.3**	16.2±3.3**
THC+*P. incarnata* benzoflavone moiety (20 mg kg^{-1})	15.7±1.61**	6.2±1.19**
P. incarnata benzoflavone moiety (10 mg kg^{-1})	–	–
P. incarnata benzoflavone moiety (20 mg kg^{-1})	–	–

Data are expressed as mean±s.d. (n = 10). THC = Δ^9-tetrahydrocannabinol. Doses of THC and the benzoflavone moiety were given twice daily, by mouth, for 6 days; SR-141716A 20 mg kg^{-1} was given by mouth on day 7. *$P < 0.05$, **$P < 0.01$ vs THC group; analysis of variance followed by Fischer's LSD test.

141716A is expected to cause abrupt cessation of cannabinoid levels in the brain which is normally manifested by a tremendous increase in locomotor activity (Cook et al 1998). Interestingly, in the present situation, SR-141716A did not alter the ambulatory behaviour of the mice receiving *P. incarnata* benzoflavone moiety +THC, upon comparing the locomotor counts recorded on the 5th day and after the administration of the cannabinoid antagonist on the 7th day as well.

An abrupt cessation of chronic treatment with

Table 4 Paw tremors and head shakes recorded for 15 min in mice 2 h after administration of SR-141716A.

Treatment	Paw tremors	Head shakes
THC group A (5 untreated mice)	140.4±6.88	29.0±1.2
THC group B (5 mice treated with *P. incarnata* benzoflavone moiety (20 mg kg^{-1})	10.0±1.0**	2.8±0.98**

Data are expressed as mean±s.d. (n = 5). THC = Δ^9-tetrahydrocannabinol. Δ^9-THC (10 mg kg^{-1}) was given twice daily, by mouth, for 6 days; SR-141716A 20 mg kg^{-1} was given by mouth on day 7 and then, in the THC-B group, the benzoflavone moiety was given by mouth 1 h later. Recordings were made 1 h after benzoflavone moiety was administered to the THC-B group. $^{**}P < 0.01$ vs THC-A group; Student's *t*- test.

cannabinoids brought about by the administration of a specific antagonist like SR-141716A is supposed to cause severe withdrawal effects which manifest in the form of paw tremors and head shakes. A perusal of Table 3 reveals that the mice receiving the *P. incarnata* benzoflavone moiety with THC chronically for 6 days had less paw tremors and head shakes as compared with the THC group. Less paw tremors and head shakes not only reflects the non-tolerant and non-dependent behaviour of mice which concurrently received the benzoflavone moiety along with THC, but also accounts for the increased locomotor activity observed in these groups of mice (Tables 1 and 2).

Finally, we also evaluated the effects of an acute single-dose administration of 20 mg kg^{-1} of *P. incarnata* benzoflavone moiety in the group of 10 mice dependent on THC, which were showing symptoms of acute withdrawal after administration of SR-141716A. This particular group was further segregated and 5 mice were given 20 mg kg^{-1} of benzoflavone moiety by mouth and the paw tremors and head shakes of the 10 mice were again recorded after 1 h. The untreated mice exhibited increased paw tremors and head shakes 2 h after the administration of the THC antagonist, whereas the 5 mice receiving an acute single dose of benzoflavone moiety (20 mg kg^{-1}, p.o.) exhibited a remarkable decrease in the number of paw tremors and head shakes (Table 4). This measurement makes us opine that even a single acute administration of *P. incarnata* benzoflavone moiety (20 mg kg^{-1}) is able to block the expression of tolerance to, and dependence on, Δ^9-THC in mice.

Thus, from this experimental study, we see the usefulness of the benzoflavone phyto-moiety of *P. incarnata* in countering Δ^9-THC dependence and tolerance. The bioactive benzoflavone moiety was able to delay the development of tolerance to the chronic administration of Δ^9-THC when administered concurrently with THC. Acute administration of the benzoflavone moiety was even able to significantly block the expression of withdrawal effects in mice rendered tolerant to Δ^9-THC, the 20 mg kg^{-1} dose of the benzoflavone moiety being highly effective.

Conclusions

In light of their tremendous abuse, cannabinoids have been put under strict legislative measures to discourage even their medicinal use, especially in the present era when the menace of drug and substance abuse has become a very serious issue for health management agencies. Since nature understands her business better than we do, plants like *P. incarnata* can offer a viable solution against the problem of cannabinoid addiction. The recently reported benzoflavone moiety can have many uses as these bio-flavonoids are very strong antioxidant, immunostimulant, anti-carcinogenic, antimicrobial and anti-anxiety agents. Benzoflavone compounds inhibit the enzyme aromatase and, thus, alter brain neurosteroids (Kellis & Vickery 1984; Merken & Beecher 2001). In this study, reversal of the effects of cannabinoids by the benzoflavone moiety is speculated to be via neurosteroidal modulation. Though these studies confirm the usefulness of *P. incarnata* in breaking down marihuana habits in accordance with traditional reports, these studies are as yet only preliminary, and the experimental design requires replication and modification to ensure that the mouse model is a good predictor for man.

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