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African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 8 Number 44 29 November, 2014

ARTICLES

Research Articles

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Full Length Research Paper

Effect of *Punica granatum* on behavior in rats

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The importance of diet in depression is now increasingly recognized, since balanced diet not only reduces the risk of various chronic diseases but also promotes normal development and functioning of all systems including brain. Hence the present study was designed to observe the effect of three doses of *Punica granatum* 2, 5 and 8 ml/kg on the behavioral changes in rats, particularly anxiolytic and antidepressant activity. During 15 days study, experimental paradigm for anxiety and depression were assessed twice using open field, elevated plus maze and forced swimming test. In open field, distance travelled, number of centre entries and number of rearing's were increased. In elevated plus maze, number of open arm entries were increased and in forced swimming test, there was decrease in duration of immobility and increase in duration of climbing. All these changes were significant at moderate and high doses of *P. granatum*. Thus it may be suggested that *P. granatum* possesses anxiolytic and antidepressant activity at moderate and high doses.

Key words: *Punica granatum*, anxiolytic, antidepressant.

INTRODUCTION

Anxiety is the most common of all mental disorders. The persistent anxiety is manifested by symptoms from at least three out of four categories that is, motor tension, characterized by muscle aches restlessness; autonomic hyperactivity, characterized by sweating, dizziness, increased heart rate; apprehensive expectation, characterized by fear and vigilance characterized by difficulty in concentration (Brown et al., 2001; Fricchione, 2004). In recent years, use of herbal remedies and dietary supplements has increased to treat mild to moderate anxiety disorders (Saeed et al., 2007). Open field test and elevated plus maze are best experimental methods for the measurement of anxiety (Herskin and Jensen, 2002; Mansouri et al., 2014).

Depression could be defined as a multifaceted condition of psychosomatic, neuroendocrine and somatic symptoms difficult to reproduce in animals (Petit-Demouliere et al., 2005), but since research in humans is limited, animal models of depression have been developed. Many symptoms of depression cannot be easily measured in laboratory rodents for example, feelings of worthlessness, suicide tendency etc., but some behavioral tests have shown to be very effective in evaluating depressive symptoms and are classically used to predict the antidepressant effect of new medications. They also provide theoretical useful models to study neurobiological and genetic mechanisms underlying depressive behavioral changes.

The existence of numerous behavioral tests to measure depression in rodents reflects the heterogeneity of

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depression like symptoms. Hence forced swimming test is the classical paradigms used to evaluate behavioral misery. Hopelessness, reported as a common trait of depression in humans, is mimicked in rodents by the paradigm of learned helplessness. Thus forced swimming test is considered to be useful for investigating depressive state in human since the behavioral immobility of animals during forced swimming has been reported to reproduce some aspects of human depression (Porsolt et al., 1977, 1978; Wilner, 1984; Yoshimura and Yamakawa, 2000).

Force swim test (FST) was than modified by different investigators (Petit-Demouliere et al., 2005; Carbajal et al., 2009), while it has also been reported that regional skin temperature changes and prolonged immobility like symptoms observed in ovariectomized mice are very identical to depressive hot-flush symptoms in women (Mori-Okamoto et al., 2004). Thus force swim test is a currently used popular model for depression due to low cost and reliability (Petit-Demouliere et al., 2005).

Pomegranate (*Punica granatum L.,* Punicaceae) is a globally consumed fruit and contains high level of phytochemicals, including polyphenols (punicalagin, ellagic acid, anthocyanins) and flavonoids (quercetin, kaempferol). *P. granatum* also contains testosterone, estrogen and estrogen like compounds (Gil et al., 2000; Langley, 2000; Kohno et al., 2004; Lansky et al., 2007; Hong et al., 2008; Wang et al., 2010; Fischer et al., 2011). These compounds have variety of activities for example, ellagic acid has anxiolytic activity (Girish et al., 2013). Progesterone has been shown to regulate anterior pituitary level of follicle stimulating hormone (Conner et al., 1999). Testosterone has been shown to have anti-depressant like activity (Buddenberg et al., 2009). Polyphenols have hypoglycemic and hypolipidemic effects (Yin et al., 2011). Flavonoids have anti-inflammatory activity (Kim et al., 2004; Garcia-Lafuente et al., 2009). However studies regarding exploratory behavior and antidepressant-like action are very rare. Thus present study was designed to evaluate the effects of orally administered *P. granatum* juice on reduced immobility during forced swim test in rats and on anxiety and motor action using elevated plus-maze and open field procedures.

MATERIALS AND METHODS

Animals

Study was carried out on adult male Wister rats with mean body weight of 220 ± 10 g. Animals were kept under controlled condition of temperature 23 ± 2°C and humidity 50 to 60% throughout the experiment in a 12/12 h light and dark cycle with free access to rat chow and tap water. Five rats were housed in each plastic cage measuring 81 × 46 × 41 cm. The use of animals during the study was in accordance with the National Institute of Health (NIH) guide for the care and use of Laboratory Animals (National Research Council, 1996) and approved by the Board of Advance Studies and Research University of Karachi.

Punica granatum Juice

The *P. granatum* (Pomegranate) was purchased from local market, identified by center of plant conservation, University of Karachi. The voucher specimen no P.G 11-12 was deposited in Department of Pharmacognosy, University of Karachi. The fruits were peeled and squeezed to yield fresh juice which was than filtered and administered through oral route in three doses that is, 2, 5 and 8 ml/kg, respectively according to body weight.

Drug treatment

All rats were divided into six groups each comprising of ten animals. One group served as control and received sterile water, three groups were treated with *P. granatum* at 2, 5 and 8 ml/kg and two other groups received standard drugs imipramine and diazepam in the dose of 25 and 3 mg/kg, respectively according to body weight (Carbajal et al., 2009). Sterile water, juices and standard drugs were administered on once daily basis for 15 days through oral route.

Open field test

Open field test (OFT) is used to assess locomotion and anxiety related behavior (Prut and Belzung, 2003) and is also the measure of exploration (Walsh and Cummins, 1976). The open field was made up of Plexi glass, 75 cm long, 75 cm wide and 40 cm high in the shape of quadratic box. Black lines were drawn on floor, which divides the floor into twenty-five (15 × 15 cm) squares. A central square (30 × 30 cm) was drawn around the middle centre squares to count number of central entries of animal. Cross section length of squares was 21 cm. The open field was located in test room, illuminated by 60 watt light bulb. All tests were recorded by camera (SONY HANDYCAM-DCR-DVD755/E) from the top view of the field. *P. granatum* juice, sterile water and diazepam were administered daily between 8.0 to 10.0 am orally for 15 days. OFT for all animal groups was performed twice on 8th and 15th day, just 1 h after administration of drugs.

Procedure

All rats received individual application and were handled by their tail. Time of exploration of field by the animal was 30 min, after which rats were returned in their home cages and arena was cleaned with 70% ethanol solution. Animals were exposed to the apparatus twice that is, 8th and 15th day of drug administration by placing into the center of the open field maze. Variables measured were, (1) distance traveled by the animal in the field by line crossing with all four paws, (2) total number of entries in central square (30 × 30 cm), when animal crossed one of the central square lines with all four paws, (3) total number rearing-frequency with which animal stood or rising up on hind legs with the forelegs against the wall of maze or in the air, (4) duration of rearing's - time spent by animal in rising up or standing position.

Elevated plus-maze

Elevated plus-maze (EPM) was used to assess the anxiety related behavior (Hogg, 1996) of *P. granatum*. Animals used in OFT underwent testing in EPM just 30 min after open field test. The EPM consisted of two open arms and two close arms placed opposite to each other, arranged around (10 × 10 cm) central platform. Open arms were 50 cm long and 10 cm wide. While close arms were 50 cm long, 10 cm wide and 38 cm high with open roof.

Table 1. Effect of *P. granatum* and diazepam on behavior of rats in open field.

Group	Days	Parameter			
		No. of rearing's	Duration of rearing's (s)	No. of centre entries	Distance travelled (cm)
Control (Sterile water)	8	50.0±5.41	200.1±47.26	4.9±0.86	2137.9±151.87
	15	51.4±5.58	207.1±48.46	5.6±0.97	2329.8±187.51
P. granatum 2 ml/kg	8	53.4±5.05	203.3±46.74	4.7±0.84	2190.0±147.83
	15	54.5±5.09	210.4±47.43	5.2±0.84	2646.9±317.49
*P. granatu*m 5 ml/kg	8	62.9±2.12*	210.0±47.54	7.2±0.69*	2786.2±382.00*
	15	63.7±2.18*	213.8±47.73	7.7±0.65*	3231.0±334.96*
P. granatum 8 ml/kg	8	63.4±2.40*	219.4±50.74	7.7±0.66*	2762.8±164.00*
	15	64.1±2.34*	220.4±50.06	8.2±0.53*	3225.3±350.31*
Diazepam	8	62.6±2.95*	235.9±48.67	7.6±0.45*	1852.3±264.28
	15	63.1±2.92*	236.5±48.72	8.1±0.37*	1983.8±264.43

n=10, Values are mean ± SEM. *P ≤ 0.05 significant as compared to control.

Procedure

Animals behavior in all test was recorded by means of a video camera (SONY HANDYCAM-DCR-DVD755/E) mounted 100 cm above the maze to have the top view. Animals were placed into the central platform, facing one of the open arms. During the test, rats were allowed to move freely in maze and explore the environment for 5 min. Variables measured were, (1) spent time in open arms, (2) spent time in close arms, (3) number of entries, when animal entered with all four paws into the open or closed arms.

Forced swimming test

Forced swimming test (FST) is used to assess depressive-like behavior. Test was done twice following administration of *P. granatum* juice, imipramine and sterile water to respective groups on 15th day 1 h just after the EPM, marked as day-1 (pre-test phase), and at 16th day, twenty-four hours after the 1st FST, marked as day-2 (test-phase).

Procedure

FST was performed in Plexiglass cylinder 46 cm tall and 20 cm wide. The cylinder had been filled with water at 25°C up to 20 ± 2 cm from bottom. The forced swim apparatus was situated in a sound isolated and dimly illuminated room. On the day-1, rats were placed individually into the water for a 15 min period. After the completion of pre-test phase, animals were removed from the water and then placed under a light heating lamp in a plastic cage for about 15 to 20 min to make them dry. Same experimental conditions of FST were applied to animals after twenty four hours that is, placed into the water for 15 min, water was replaced after each animal testing. All tests were recorded by camera (SONY HANDYCAM-DCR-DVD755/E) from the front view of the whole cylinder. Variables measured were, (1) duration of immobility that is, time at which animal showed lack of motion, except only those movement necessary to keep his head above water, (2) duration of swimming that is, time at which animal showed vigorous movements with fore paws in water, (3) duration of climbing that is, time at which animal showed vigorous movements with fore paws and hind paw along the walls of cylinder (Buddenberg et al., 2009).

Statistical analysis

All data was expressed as the mean ± standard error of mean (SEM), data was analyzed using superior performance statistical software (SPSS) version 20. ANOVA followed by post hoc was performed for comparisons of values with control. Values of $p \leq 0.05$ were considered statistically significant and $p \leq 0.005$ highly significant.

RESULT

Table 1 shows the effect of *P. granatum* on behavior of rats in open field. There was significant increase in No. of rearing's, No. of centre entries and distance travelled at 5 and 8 ml/kg than control groups on respective days. While no significant change was observed in any parameters at 2 ml/kg than control. Animals received diazepam showed significant increase in number of rearing's and central entries on both 8th and 15th day than as control. Table 2 shows the effect of *P. granatum* on behavior of rats in elevated plus maze. There was significant increase in number of entries in open arm but no significant changes were observed in time spent on open arm, close arm and no. of entries in close arm at 5 and 8 ml/kg both on 8th and 15th day than control. However no significant changes were observed in any parameters at 2 ml/kg than control. Animals received diazepam showed significant increase in number of entries in open arm and

Table 2. Effect of *P. granatum* and diazepam on behavior of rats in elevated plus maze.

Groups	Days	Parameter			
		No. of entries in open arm	Time spend in open arm (s)	No. of entries in close arm	Time spend in close arm (s)
Control	8	3.5±0.70	125.0±23.0	6.5±0.76	175.0±23.0
	15	4.1±0.73	140.0±24.8	5.7±0.84	160.0±24.8
P. granatum 2 ml/kg	8	5.0±0.81	122.0±20.48	5.6±1.13	178.0±20.48
	15	5.1±0.79	138.0±22.64	5.1±1.03	162.0±22.64
P. granatum 5 ml/kg	8	6.2±1.04*	101.0±22.03	6.1±1.10	199.0±22.03
	15	7.1±0.90*	132.0±26.02	6.8±0.91	168.0±26.02
P. granatum 8 ml/kg	8	6.7±1.08*	114.0±21.86	6.2±1.08	186.0±21.86
	15	7.0±1.01*	126.5±25.10	6.5±1.14	173.5±25.10
Diazepam	8	6.6±0.66*	157.0±17.9	3.2±0.55*	143.0±17.9
	15	7.0±0.85*	210.0±16.3*	2.4±0.60*	90.0±16.3*

n=10, Values are mean ± SEM. *P ≤ 0.05 significantly different as compared to control.

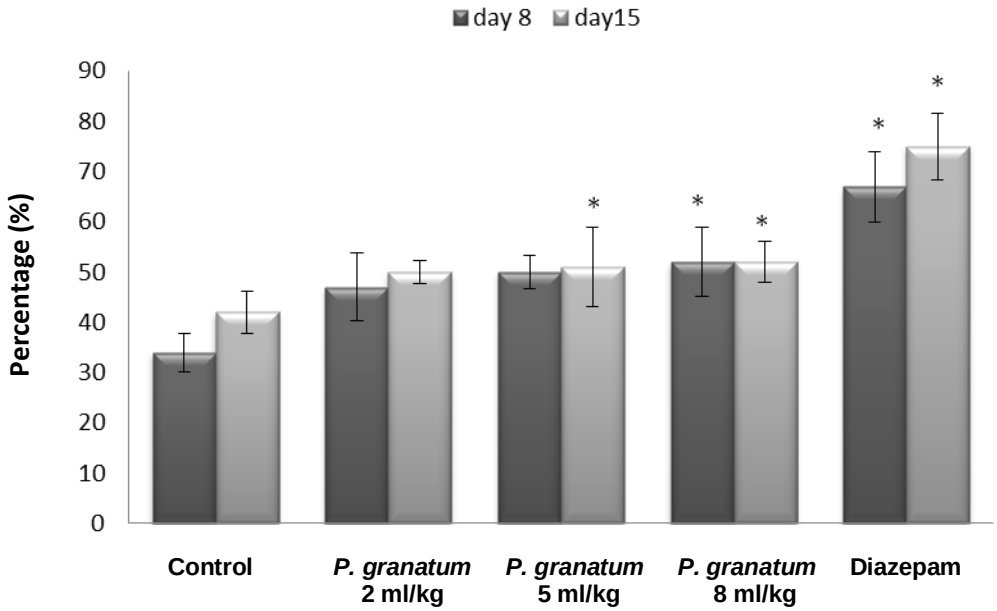

Figure 1. Percentage of entries into the open arm in Elevated Plus Maze. mean ± S.E.M% of open arm entries (percent increase from control). *P ≤ 0.05 significantly different as compared to control.

and time spent in open arm, while there was significant decrease in number of entries in close arm and time spent in closed arm than control.

Figure 1 shows percentages of entries in to the open arm by control, *P. granatum* at all three doses and diazepam in elevated plus maze. Percentages at 8th day were 34, 47, 50, 52, 67% and at 15th day percentages were 42, 50, 51, 52 and 74% of control, 2, 5, 8 ml/kg and diazepam, respectively. Change in percentage in 8 ml/kg and diazepam group at both days and 5 ml/kg at day 15 were significantly increased as compare to control. Figure 2 shows percentages of time spent in the open arm by control, *P. granatum* at all three doses and diazepam in elevated plus maze. Percentages at 8th day were 42, 41, 34, 38, 52% and at 15th day 47, 46, 44, 42, 70% of control, 2, 5, 8 ml/kg and diazepam, respectively. Change in percentage in diazepam group at both days was significantly increased as compare to control.

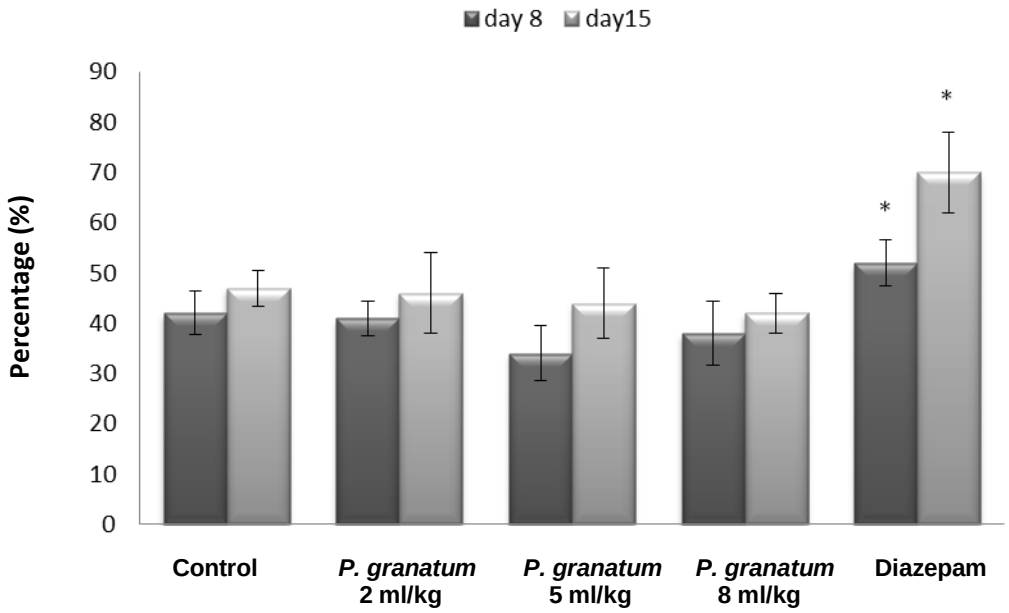

Figure 2. Percentage of time spent in the open arm in elevated plus maze. Mean ± SEM% of time in open arm (percent increase from control). *P ≤ 0.05 significantly different as compared to control.

Table 3. Effect of *P. granatum* and imipramine on behavior of rats in forced swimming test

Groups	Day	Parameters		
		Immobility duration (s)	Climbing duration (s)	Swimming duration (s)
Control	1	151.5±13.39	67.4±8.83	681.1±12.56
	2	160.5±21.60	66.4±6.43	673.1±18.20
P. granatum 2 ml/kg	1	116.3±03.69	82.6±3.09	701.1±02.69
	2	122.3±04.91	84.6±4.26	693.1±05.58
P. granatum 5 ml/kg	1	105.1±16.32*	97.4± 10.58*	697.5±17.24
	2	108.1±16.89*	96.4±12.45*	695.5±18.34
P. granatum 8 ml/kg	1	105.5±03.60*	98.40±06.58*	696.1±08.86
	2	109.5±04.77*	93.7±07.00*	696.8±09.97
Imipramine	1	89.1±06.88**	71.0±08.66	739.9±14.02*
	2	87.7±08.91**	68.1±03.01	744.2±47.17*

n=10, Values are mean ± SEM. *P ≤ 0.05 significantly different as compared to control. **P ≤ 0.005 highly significant as compared to control.

Table 3 shows the effect of *P. granatum* on behavior of rats in forced swimming test. There were significant decrease in duration of immobility and significant increase in duration of climbing at 5 and 8 ml/kg during first and second exposure to forced swimming than control. While highly significant decrease in duration of immobility and significant increase in duration of swimming were observed by imipramine during first and second exposure of forced swimming test than control. However no significant changes in duration of immobility, duration of climbing and duration of swimming were observed at 2 ml/kg on both exposures as compare to control.

Figure 3 shows percentage decrease in immobility duration by control, *P. granatum* at all three doses and imipramine in force swimming test. Percentages during first exposure were 17, 14, 11, 11, 10% and during the second exposure percentages were 18, 15, 12, 13, 10% of control, 2, 5, 8 ml/kg and imipramine, respectively. Decrease in percentage in 5, 8 ml/kg and imipramine group at both exposure were significant as compare to control. Figure 4 shows percentage increases in climbing duration by control, *P. granatum* at all three doses and imipramine in force swimming test. Percentage during first

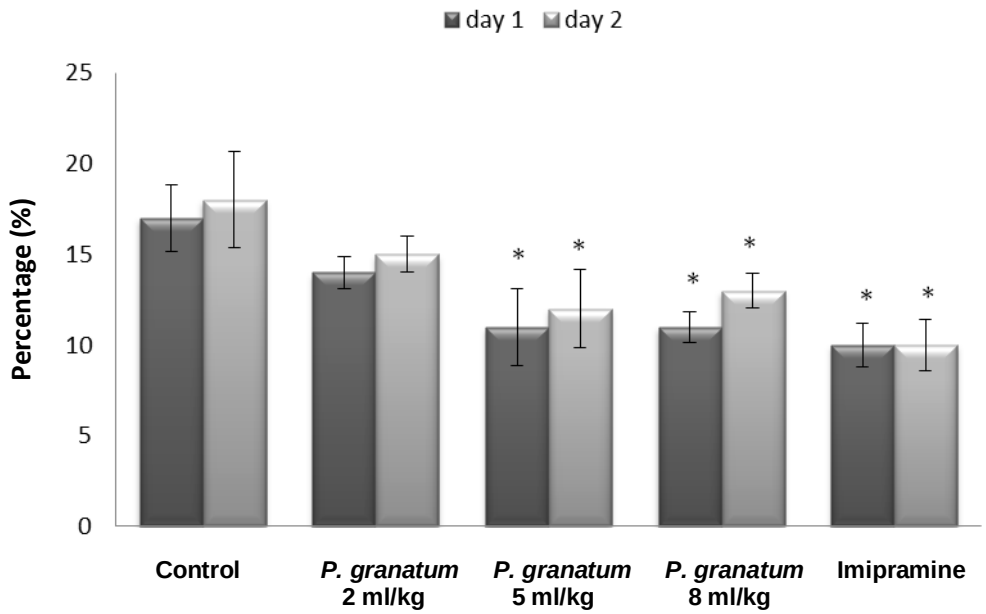

Figure 3. Effect of *P. granatum* and imipramine on immobility inhibition in FST. Mean ± SEM, inhibition immobility (percent decrease from control).
*P ≤ 0.05 significantly different as compared to control.

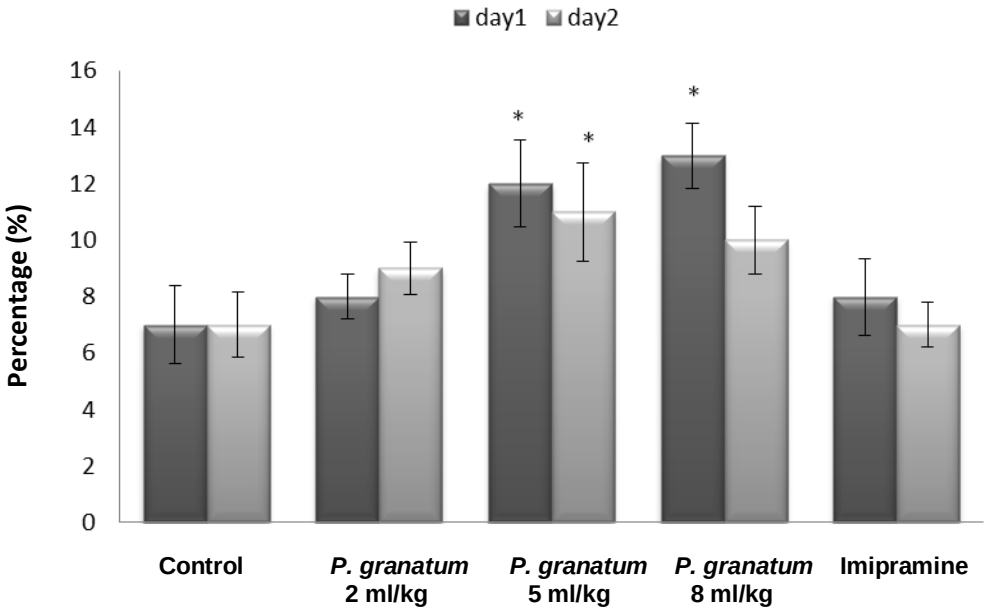

Figure 4. Effect of *P. granatum* and imipramine on percentage of climbing duration. Mean ± SEM, increase climbing duration (percent increase from control).
*P ≤ 0.05 significantly different as compared to control

first exposure were 7, 8, 12, 13, 8% during first exposure and 7, 9, 11, 10, 7% during the second exposure of control, 2, 5, 8 ml/kg and imipramine, respectively. Increase in percentage in 5 and 8 ml/kg group at both expo-sure were significant as compare to control. Figure 5 shows percentage increases in swimming duration by control, *P. granatum* at all three doses and imipramine in force swimming test. Percentages during first exposure were 76, 78, 77, 76, 82 and 75, 74, 76, 77 and 83% during the second exposure by control, 2, 5, 8 ml/kg and imipramine, respectively. Increase in percentage in imipramine at both exposures was significant as compared

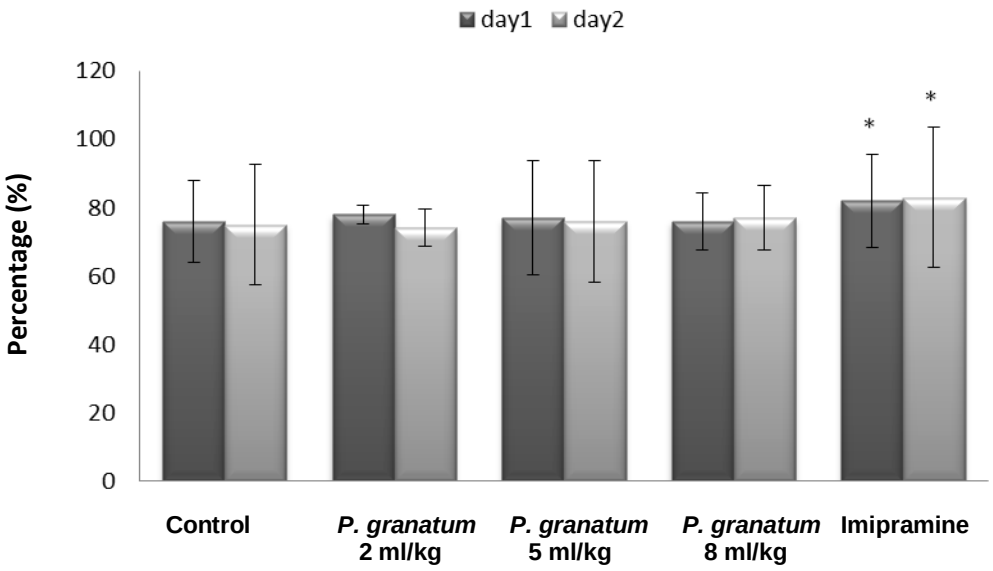

Figure 5. Effect of *P. granatum* and Imipramine on percentage increase in swimming duration Mean ± SEM, increase swimming (percent increase from control). *P ≤ 0.05 significantly different as compared to control

to control.

DISCUSSION

In present study *P. granatum* was tested for its spontaneous motor activity, anti-anxiety and anti-depressant actions in healthy male rats. These behavioral changes were studied using open field test, elevated plus maze and forced swimming tests. *P. granatum* was administered in three different doses 2, 5 and 8 ml/kg. Turk et al. (2008) used *P. granatum* juice in the dose of 1 ml and Patel et al. (2008) assessed safety of the juice. Punicalagin, major water soluble ellegatannins of *P. granatum*, found in its juice vary from 1500 to 1900 mg/L and its daily intake of 6 oz/60 kg individual weight may range from 4.4 to 5.6 mg/kg (Patel et al., 2008).

Open field test (OFT) and elevated plus maze (EPM) were used to evaluate anxiolytic effect, as behavior animal models (Mansouri et al., 2014). In OFT the 5 and 8 ml/kg showed significant increase in number of centre entries, number of rearing's and distance traveled both on 8th and 15th day as compared to control. The behavior of increase in number of centre entries, number of rearing's at 5 and 8 ml/kg were comparable to that of diazepam treated group. These observed effects of *P. granatum* may be due to its increase spontaneous, locomotor and anxiolytic activity, since the number of central square entries is the measure of exploration behavior and anxiety. A high frequency/duration of these behaviors indicates high exploratory behavior and low anxiety levels (Walsh and Cummins, 1976; Brown et al., 1999; Bailey and Crawley, 2009). The spontaneous activity was measured by distance traveled and rearing's. The rearing is an index of locomotor activity (Alves et al., 2005), while increase in distance traveled is an indication of increase in motor activity as well as stimulation of central nervous system (Kennett et al., 1987; Czech, 2002). *P. granatum* significantly increased the number of centre entries, rearing's and distance travelled. This stimulation in motor activity can be interpreted in term of central nervous system (CNS) stimulant effect.

In EPM *P. granatum* showed increase in total entries at all three doses; however significant increase in number of entries in open at 5 and 8 ml/kg both on 8th and 15th day was observed. This increase in number of entries to open arm were quite similar to standard drug diazepam. Comparison between parameter "no. of entries in open arm" with "no. of entries in close arm" 5, 8 ml/kg and diazepam group on both 8th and 15th day showed more no. of entries in open arm as compared to close arm. While comparison between the parameter "time spend in open arm" with "time spend in close arm" only diazepam treated group showed more time spent in open arm as compared to the close arm. All mentioned significant changes at 5 and 8 ml/kg are comparable to diazepam group. Thus it could be suggested that the increase are likely be due to general stimulatory activity. Hence, anti-anxiety behavior can be determined by measuring spontaneous motor activity that is, total or open arm entries (Pellow and Sandra, 1986; Budzynska et al., 2013; Mansouri et al., 2014).

In EPM, data of percentages of total activity gives us a picture of differences in locomotor activity between control, treated and diazepam groups. Among treated group 5 and 8 ml/kg showed highest locomotor activity

that could be compared with diazepam group. Increases in locomotor behaviors are the measurement of CNS stimulant activity and decreased anxiety-like behavior. All these behavioral changes of *P. granatum* can be taken in terms of CNS stimulant and anxiolytic effect. Since there is no way to confidently differentiate a drug effect as either stimulant or anxiolytic due to a similar phenotype, and there certainly can be an overlap in terms of underlying mechanisms. Hence increase in distance traveled is an indication of increase in motor activity as well as stimulation of central nervous system (Kennett et al., 1987; Czech, 2002) and number of rearing's is an index of locomotor activity (Alves et al., 2005). Thus, forced swimming test (FST) is considered to be useful for investigating depressive state, since the behavioral immobility of animals during forced swimming has been reported to reproduce some aspects of human depression (Porsolt et al., 1977, 1978; Wilner, 1984; Yoshimura and Yamakawa, 2000). Duration of immobility is used to quantify overall anti-depressant or depressive like effect of the drugs (Taiwo et al., 2012). Present study also characterized the effect of the *P. granatum* on rats in FST following 15 day treatment. To obtain full antidepressant effect, rats were subjected to forced swimming at the end of dosing period that is, 15th and 16th day, and was considered as day 1 and day 2, respectively (Buddenberg et al., 2009). Hence it is critical to perform FST on repeated administration in rat model (Mora et al., 2005).

Result of the present study reveals that 5 and 8 ml/kg treated group significantly reduce the duration of immobility, which is comparable to that of imipramine. 5 and 8 ml/kg treated group also showed significant increase in duration of climbing, as a compensatory mechanism of reduction in immobility, whereas imipramine showed significant increase in duration of swimming. Decrease in immobility induced by imipramine like drugs is generally accompanied by increase in swimming, whereas climbing duration was not affected by these drugs (Barros and Ferigolo, 1998). Comparison between parameter "immobility duration" with "climbing duration" 2 ml/kg treated group immobility duration is much greater than climbing duration, whereas in case of 5, 8 ml/kg and imipramine treated group on both day 1 and 2 showed less differences. Percentages of reduction in immobility at 5 and 8 ml/kg were also quite similar to imipramine. This comparison reveals similar response of these two doses of *P. granatum* with that of the response of standard drug imipramine. Hence 5 and 8 ml/kg revealed a trend, indicating an antidepressant like effect.

Comparison of "day 1" with "day 2" *P. granatum* treated rats showed reduction in mobility and increase in climbing behavior at day 1, the first FST exposure as compare to day 2 second FST exposure. This possibly may be due to the reduced flavonoids level in body, since flavonoids absorbed from the gastrointestinal tract and excreted either unchanged or as metabolites in the urine or feces (Cook and Samman, 1996; Sousa et al., 1996). Result of the present study showed significant decrease in the duration of immobility in FST, increase in the distance travelled, number of central entries in OFT and number of entries in open arm in EPM by *P. granatum* in dose dependent manner, suggesting the need to work on higher dose, to obtain desired effects. The antidepressant action might be due to the presence of flavonoid (Ohlsson et al., 2010). Major flavonoid found in *P. granatum* is ellagic acid, since ellagic acid is reported to produce anxiolytic action (Girish et al., 2013). These results of *P. granatum* may be due to presence of testosterone in it (Kim et al., 2004). Since there is evidence that testosterone could attenuate immobility in healthy rats and act as antidepressant by increasing central dopaminergic and 5-hydroxytrptaminergic metabolism (Buddenberg et al., 2009). Another study suggests that anti-depressant like effect might be due to the estrogen which is an important component of *P. granatum* (Kim et al., 2002). Hence, role of estrogen or estrogen like compound as anti-depressant have been well documented (Mori-Okamoto et al., 2004).

From the results of the present study, it may be concluded that *P. granatum* is most effective in moderate and high doses for its CNS stimulant, anxiolytic and antidepressant effects. However further studies on more different doses are required to reveal the role of essential substances in all their observed effects.

Conflict of interest

Authors declare that they have no competing interests.

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Full Length Research Paper

Some physical properties of novel *Cannabis* suppositories formulated with theobroma oil

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As part of development efforts for a suitable dosage form, crude *Cannabis* resin was formulated into suppository dosage form using theobroma oil and the physical properties of the suppositories were evaluated. The following physical properties were evaluated: appearance (texture, presence or absence of entrapped air, contraction holes), liquefaction time, uniformity of weight and *in-vitro* release profile of the crude marijuana resin from the suppositories. The torpedo shaped suppositories were smooth in texture with absence of entrapped air and contraction holes. The suppositories had uniform greenish brown colour and low weight variation. The liquefaction time was also low. The 300 mg *Cannabis* crude in 4 % Tween 85 showed highest melting time (11.67 ± 057 min) while the incorporation of Tween 85 improved the release profile (0.0452-0.0650 %) in different batches. It is possible to formulate marijuana suppositories with satisfactory physical properties; however, release profile of marijuana from the suppository bases was generally low even though the addition of Tween 85 greatly enhanced drug release.

Key words: Crude *Cannabis* resin, sustained release, liquefaction time, weight uniformity, release profile.

INTRODUCTION

There are anecdotal reports of *Cannabis* relieving the sign and symptoms of various disease conditions such as asthma, convulsion, multiple sclerosis (MS), ocular pressure, acute post-operative and intractable pain, as well as stimulating appetite and antispasmodic (Russo, 2011; Ben, 2006; Hazekamp and Grotenhermen, 2010; Noyes et al., 1975; Wade et al., 2003; Grant, 2001; Tomida et al., 2006; Formukong et al., 1988; Obonga, 2006; Regelson et al., 1976; Di Tomaso et al., 1996). Other medicinal values such as antiemetic and use in palliative or terminal care have been reported for inhaled *Cannabis* and oral tetrahydrocannabinol (THC) (Matsuda

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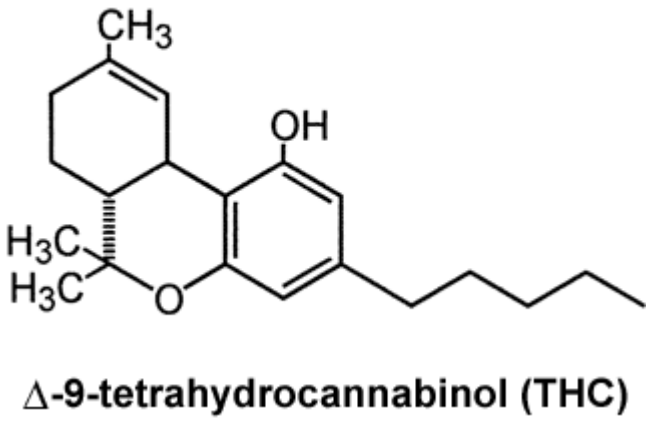

Figure 1. Chemical structure of marijuana

et al., 1990; Vincent et al.,1983; Salan et al., 1979; Vinciguerra et al., 1988; Consroe et al., 1996; Elsohly et al., 1985; Holister, 1971). Health hazards associated with *Cannabis*-based medicines are largely as a result of the difficulty that physicians encounter in obtaining consistent dose from batches of plant material of varying potency (Gierienger, 1999) and due to possible pathogens and microtoxins present in the *Cannabis* (Kagen et al., 1983; Taylor et al., 1982; Gordon et al., 2013). Consequently, patients suffer from ineffective (under) dose or the unwanted intoxication effects resulting from an over dose (British Medical Association, 1997). Modern techniques have attempted to solve this problem of quality control in the *Cannabis* phyto-medicines through plant breeding and cultivation. However, the issue of narrow therapeutic window between the desired benefits and the usual unwanted psychoactive effects remains a challenge (Institute of Medicine, 1999).

∆-9-Tetrahydrocannabinol, Δ^9-THC (Figure 1) (Grotenhermen, 2002), presently the most widely used and the major psychoactive constituent of *Cannabis*-based medicine, can be taken orally (Brennesisen, 2002) but absorption of THC from this oral route is very low and unreliable, especially when compared with the non-conventional method of smoking or inhaled by vaporization. Inhalation of *Cannabis* is a very efficient way of delivering the drug quickly and in manner that allows flexible dose titration (British Pharmacopia, 2001). Smoking, however, carries serious medical risks; the irritant effects of *Cannabis* smoke can lead to bronchitis and later to far more serious hazards such as lung cancer and many other bronchial diseases (Ashton, 2001).

In line with current trend in the scientific world, researches have shifted from isolation of pure active ingredients and structural determination and eventual synthesis of active compounds to formulation of the crude drugs. Experience has shown that pharmacological activity may not be resident in any of the components of the phyto-constituents but in the *Cannabis* resin as a whole. The current challenge in the medicinal application of *Cannabis* is therefore, the development of suitable dosage forms which would enhance the stability, convenience of administration and bioavailability of the drug.

Previous reports indicated efficient delivery of *Cannabis* and its derivatives to systemic circulation in formulations using different lipid carriers (Russo, 2002; Grant et al., 2012; Mattes et al., 1993). We realized that oral bioavailability of THC need not be low if there is a suitable lipid carrier, that smoking is not particularly efficient for its delivery from a pharmacokinetic standpoint, and that titration is hardly easy, particularly with modern Western strains of high potency, which tend to produce maximum psycho-activity with absorbed doses far in excess of those needed for medical symptom control. Hence the present study was aimed at evaluating some of the physical properties of theobroma oil-based suppositories containing crude *Cannabis* resin in a lipid carrier as part of formulation development process.

MATERIALS AND METHODS

Hydrochloric acid (BDH, England), theobroma oil (BP Pharmaceutical Grade), Tween® 85 (BDH, England) and crude *Cannabis* resin (extracted in our laboratory). Other reagents and solvents were of analytical grade and were used as such without purification.

Source and identity of plant materials

The fresh whole leaves of *Cannabis sativa* were collected and the plant was identified by a plant Taxonomist at the Crude Drug and Research Unit of the National Drug Law Enforcement Agency (NDLEA) Enugu, Enugu State of Nigeria.

Preparation of plant extracts

Whole leaves of *C. sativa* were rinsed thoroughly with purified water, shade-dried in open air for 48 h and pulverized to coarse powder. One thousand grams (1000 g) of the powdered leaves of *C. sativa* L. was extracted with 2.5 L of methanol (95% v/v) for 8 h using a soxhlet extractor (Gallenkamp, England). The crude methanolic extract was evaporated to dryness under reduced pressure, using a rotary evaporator (Gallenkamp, England) at an optimum temperature of between 40 and 45°C, to yield 173.25 g of crude resin tar.

Preparation of *Cannabis* formulations

Preparation of suppositories

Using the displacement value of 1.5 for theobroma oil, the correct quantity of the base for each batch was calculated. Six batches of the suppositories (three batches contained 300 mg of *Cannabis* crude resin with 2, 4 and 6% Tween® 85 and three batches of 300, 600 and 900 mg, respectively of crude *Cannabis* resin per suppository without Tween® 85 were prepared. Enough quantities to yield 12 suppositories per batch were calculated at each instant.

Table 1. Results of the physical parameters of the *Cannabis* crude resin extract suppositories

Batch	Parameters		
	Weight uniformity (g ± CV)	Liquefaction time (mean ± SD)	Absolute drug content (mg in Tween ® 85)
1	1.08 ± 2.91	11.00 ± 3.60	300 in 2% Tween® 85
2	1.04 ± 3.05	11.67 ± 0.57	300 in 4% Tween® 85
3	0.96 ± 6.54	10.00 ± 1.00	300 in 6% Tween® 85
4	1.04 ± 2.45	9.33 ± 0.57	300
5	1.04 ± 3.79	8.67 ± 0.57	600
6	1.09 ± 1.69	7.43 ± 1.25	900

CV = Coefficient of variation, SD = Standard deviation

The correct quantity of the drug was added to the base (after melting), with continuous stirring until it was cool but pourable. The preparation was poured into a 1.0 g mould (previously lubricated with glycerin) until there was an overflow and then cooled at 0°C for 30 min. After cooling, the suppositories were removed from the mould and stored in the refrigerator for further experiments.

Evaluation of suppositories

Appearance

Two suppositories were randomly selected from each batch and the external and internal surfaces when cut longitudinally examined with the naked eye and also with a hand lens. The suppositories were examined for the presence or absence of air bubbles, brittle fracture, uniformity of mixing and for presence or absence of contraction holes.

Uniformity of weight

Six suppositories were picked at random and weighed together using a torsion balance. They were also weighed individually and the mean, variance, standard derivation and coefficient of variation calculated.

Liquefaction time

Liquefaction time apparatus, which can also be used to determine the melting point of fatty base suppositories proposed by Setnikar and Fantelli (1962) was modified and used in this study. Each suppository was placed in a heat-resistance and inelastic polyethylene material and tied directly on the bulb of a thermometer using an in-extensible thread. The thermometer with suppository was inserted into a 0.1 N HCl solution maintained at 37 ± 0.1°C by means of a thermostatic heating mantle (Jurgen & Co.). The time taken for the suppository to melt at that temperature was recorded. Average of four determinations was taken as the liquefaction time.

Construction of calibration curve (Beer's plot)

Cannabis crude resin (100 mg) was weighed out and dissolved in 100 ml solution of ethanol to obtain a stock solution. From the stock solution, 0.1 ml was diluted to 100 ml with ethanol (concentration 0.1 mg %). Similarly, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 to 2.0 ml of the stock were diluted to 100 ml to obtain the corresponding strengths in mg %. The absorbance of each diluted sample was determined at 274 nm using a UV/Vis spectrophotometer (UV 2102, Unico, USA). The absorbance values were plotted against the concentration to yield Beer's plot. The slope of the graph was determined. Validation of the method was performed to ensure that the calibration curve between 1 and 20 µg/ml was in the linearity range of the assay and the coefficients of variation were less than 2% both intra-day and inter-day.

Release studies

The Erweka dissolution test apparatus (Erweka, Germany) was used for the determination of the release rate of the suppositories. Each suppository was placed in the appropriate compartment of the dissolution apparatus containing 400 ml of 0.1 N HCl buffer solutions. The paddle was rotated at 120 rev/min and the dissolution medium was maintained at 37 ± 1°C. At predetermined time intervals, 5.0 ml samples were withdrawn and appropriately diluted. A 5.0 ml quantity of buffer solution (pre-warmed to the sink temperature of 37 ± 1°C) was added to the dissolution medium at each time interval to compensate for the sampling and maintain the sink conditions. The absorbances of the dilute solution were measured at 274 nm with a spectrophotometer, and the concentrations were determined from the calibration curve. Average of two-absorbance readings at each time interval was used for all batches.

RESULTS AND DISCUSSION

The suppositories were torpedo-shaped, smooth in texture with absence of entrapped air, contraction holes or brittle fracture. The external and internal surfaces of the suppositories were uniform in appearance when examined with the naked eye and hand lens. The uniformity in appearance was in terms of colour (greenish brown) and texture. This indicates satisfactory subdivision and dispersing of suspended material and all the batches passed the test according to British Pharmacopoeia (BP) specifications (British Pharmacopeia, 2001). The suppositories had uniform weights as shown in Table 1 (with the exception of batch 3). The coefficients of variations

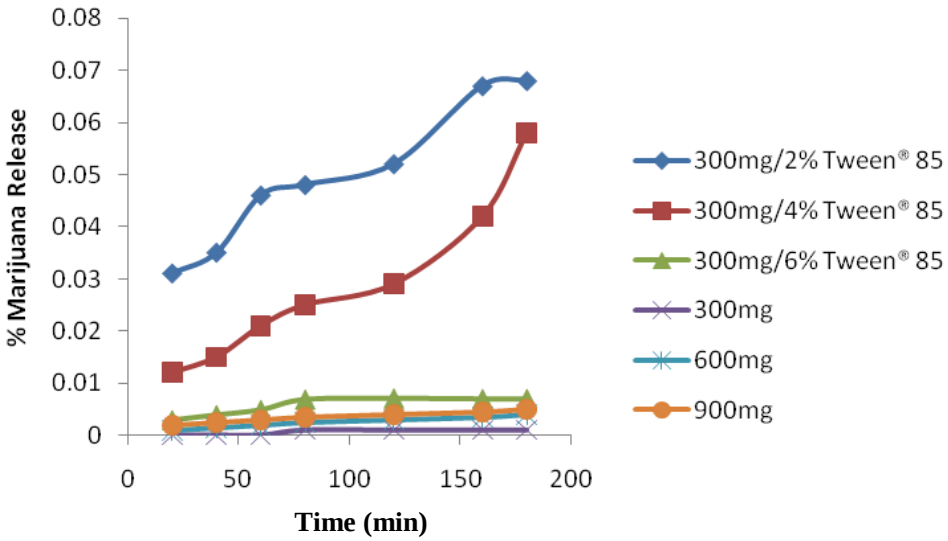

Figure 2. Release profile of crude *Cannabis* from the suppositories in 0.1 N HCl

of variation (CV) were low for all the batches. This indicates that the mixture of ingredients and the suppository base was fairly homogenous before pouring into moulds. Also, the variation in weight of the suppositories may have resulted from sedimentation of drug during pouring since the drug (that is *Cannabis*) was only dispersed in the base. All the batches (with the exception of batch 3) passed the test according to BP specifications (British Pharmacopeia, 2001). The liquefaction times were generally low. The knowledge of the liquefaction time is essential because a suppository which takes too long to liquefy may be expelled before liquefaction occurs together with the drug it contains. Besides, liquefaction time is analogous to disintegration time of tablets. A drug formulation that does not liquefy easily may be expelled before drug release occurs and may also exert a mechanical irritant action on the ampulla even if the base and the drug, per se are not irritant (Setnikar and Fantelli, 1962). The 300 mg *Cannabis* crude in 4% Tween 85 showed highest melting time (11.67 ± 057 min). This may be attributed to the heat resistance of Tween® 85, which modified the liquefaction of theobroma oil.

Figure 2 shows the release profile of *Cannabis* crude resin from the suppositories. The release of *Cannabis* crude was enhanced in batches 1 and 2 containing 2 and 6% of (0.065 and 0.0452% at 150 min) Tween® 85, respectively, while batches 4, 5 and 6% releases (0.0030, 0.0045 and 0.0057%, respectively at 150 min) were very low. This may be as the result of incorporation of various percentages of Tween® 85 into batches 1 to 3, while batches 4 to 6 had no Tween® 85 incorporated into them. It might be said that the release profile of *Cannabis* from the suppository bases was generally low even though the addition of Tween® 85 greatly increased drug release. While the results of this preliminary study appear promising, our team is currently on researches to ascertain the *in vivo* performances of the *Cannabis* suppositories in experimental animals and then humans to determine the levels of THC or cannabinoids in serum using modern analytical tools.

Conclusion

It is possible to formulate *Cannabis* suppositories with satisfactory physical properties using theobroma oil. The incorporation of polysorbate 85 increased *Cannabis* release from the suppository cavities by virtue of its ability to lower liquefaction time of the suppositories. However, the release rate was generally low from the suppositories, indicating sustained release potential.

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Conflict of interest

Authors report no conflict of interests.

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