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Antioxidant Potential of *Phoenix dactylifera* Linn Extract and its Effects on Calcium Channel Antagonist in the Treatment of Withdrawal Syndrome in Morphine Dependent Rats

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ABSTRACT

Date fruit *Phoenix dactylifera* (Linn) is remarkably known for its nutritional and numerous health benefits that are linked to its wide variety of bioactive compounds. Calcium channels were reported to play an important role in the mechanism of morphine dependence and withdrawal syndrome. This study examined the antioxidant potential of date fruit extract on calcium channel antagonists in the treatment of withdrawal syndrome in morphine-dependent rats. A total of thirty six (N=36) *Sprague-Dawley* adult male rats (weight 200-220 g) were used in this study. Morphine dependence was induced by subcutaneous injection of an increasing dose of morphine (ascending from 10 to 60 mg/kg) twice daily over a period of 5 days. The withdrawal syndrome was precipitated by administration of naloxone (*i.p*) 2 hours after the last morphine injection. For chronic study, the extracts or nifedipine were administered 30 minutes prior to each morphine injection, whereas the extract or nifedipine was only administered one hour after last morphine injection in acute studies. The rats were observed for the presence of withdrawal signs (jumps, tremor, eye ptosis, teeth chattering, wet dog shaking, diarrhoea and urination). The treatments suppressed withdrawal syndrome in morphine dependent rats. A statistically significant difference was observed between chronic and acute administration of extracts and nifedipine (p<0.001). The suppression of naloxone precipitated morphine withdrawal by the extracts possibly occurred via calcium channel blockage and the reversal of neuronal adaptation by its phenolic compounds. Therefore, *Phoenix dactylifera* could serve as an appropriate treatment of opioid addiction.

Keywords: Antioxidant, Calcium channel antagonist, Morphine withdrawal syndrome, *Phoenix dactylifera*.

Introduction

Opioid addiction and withdrawal syndrome are part of the global health concerns and contribute significantly to the existence of social and communal vices such as the rise in the rates of divorce, unemployment and government expenditure on addiction related legal and medical issues.[1] Opioids such as morphine are widely used in the clinical management of pain. Their clinical usefulness, however, is limited by tolerance and dependence. A detailed understanding of molecular mechanisms of morphine tolerance and physical dependence is considered to be essential for treatment and prevention of this phenomenon. However, many drugs with different pharmacological mechanisms of action have been tested for their effects on morphine tolerance and physical dependence.[2-4] None of these drugs have been established to be completely effective and free of adverse effects.[5] Dependence could be described as an altered physiological state caused by repeated opioid exposure such that cessation of drug use leads to a withdrawal syndrome characterized by serious physiological disturbances and emotional symptoms. The latter is perhaps the strongest determinant of opioid addiction.[6,7] Addiction can be defined as compulsive urge loss of control over drug use, regardless of adverse effects and its consequences.[1,8] Among the various mechanisms implicated in management of morphine dependence, voltage-dependent calcium channels have offered the most hopeful breakthrough.[2] Similarly, the past few decades have recorded sustained rise in the understanding of the role of calcium channel blockers in the central nervous system and management of opioid drugs addiction.[9-10] It has been shown that modifications in Calcium ions fluxes seem to play a basic role, not only in the acute effects of morphine, but also in the manifestations of morphine withdrawal syndrome.[10]

Nowadays medicinal plants continue to provide valuable therapeutic tools in both modern and in traditional medicine.[1] *Phoenix dactylifera Linn* (Date palm), a member of Arecaceae family. It is termed "Nakhla" by the Arabs, meaning Tree of life. It is popularly cultivated in the Middle East since 600 BC. It is native to the countries around the Arabian Gulf.[12] Due to its high phenolic content, various parts (fruit, seed leave and pollen grain) of *P. dactylifera* (PD) are widely used in the treatment of numerous disorders, including memory disturbances, fever, inflammation, paralysis, loss of consciousness and nervous disorders.[13,17-19] Studies have also substantiated the possession of

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numerous pharmacological activities by PD.[13] It exerts protective effect against hypertension and coronary heart disease. Similarly, it possesses anticancer, antioxidant, hepatoprotective, antiulcertavie, anti-inflammatory, antiproliferative, antimutagenic, antibacterial, antifungal and antiviral activities.[14-16] Studies reported that antioxidant activity of *P. dactylifera* is attributable to the presence of phenolic compounds.[17-19] Furthermore, many studies have described the therapeutic effects of phenolic compounds as calcium channel antagonist.[20,21] This study determined the total phenolic contents (TPC) of *P. dactylifera* extracts and assessed its effect on calcium channel antagonist in morphine dependent rats toward identification of novel non-opioid treatment for addiction.

Materials and Methods

Chemicals and Reagents

Methanol, ethanol, analytical grade distilled water, gallic acid, quercetin, Folin-Ciocalteu's reagent, sodium carbonate and aluminum chloride. All chemicals were purchased from Sigma-Aldrich. The morphine sulphate (10 mg/mL ampoule) and naloxone hydrochloride (0.4 mg/mL ampoule) were obtained from Universiti Sultan Zainal Abidin (UniSZA) Medical Centre (UMC), Malaysia.

Extracts preparation

P. dactylifera was purchased from a local supermarket in Terengganu, Malaysia. The fruit was authenticated by a Botanist at Faculty of Bio-resource and Food Industry, UniSZA with the voucher number: 00289. Then the fruits were washed and separated into pit and flesh. The flesh was further cut into pieces, oven dried and pulverised. 100 g of powdered fruit was weighed and placed in 1000 mL conical flask, macerated with 500 mL of solvents [methanol (95 %), ethanol-water (v/v 50/50) and distilled water] and placed for 72 hours in a dark cupboard with vigorous shaking at intervals. The mixture was then filtered using Whatmann No 4 filter paper and the filtrate was concentrated using rotatory evaporator. The concentrated extracts were then placed in the oven at 40 °C to allow for complete evaporation. All the three extracts [Date methanol extract (DME), Date ethanol-water (DEW) and Date aqueous (DA)] were stored at -20°C until use.

Determination of Total Phenolic Content

Total phenolic content was determined following previously described technique.[22] Briefly, 0.25 mL of 1 mg/mL sample was pipetted into a test tube followed by addition of Folin-Ciocalteu's reagent (1:10 dilution), the mixture was then incubated for 2–5 minutes. 1 mL of Sodium carbonate (7.5 %) was added to the mixture and incubated for 30 minutes. The absorbance of the colour formed was measured at 760 nm against blank sample. The measurements were compared with standard curve of prepared gallic acid solutions (250, 125, 62.5, 31.25, 15.63 and 7.82 µg/mL). All measurements were performed in triplicate. The Total phenolic contents of the fruits extracts were expressed as mg of gallic acid equivalents (GAE)/g of extracts.

Animals

The study was conducted in adherence to approved institutional animal care and use guidelines issued by UniSZA Animal Ethic Committee (UniSZA/AEC/14/005). Adult male Sprague-Dawley rats (2 to 3 months age) weighing 200-220 g, were obtained from the animal facility of the Institute. The animals were housed (n=4) under controlled condition of ambient temperature (22 ± 2°C), photoperiod controlled room (light: dark: 12 h: 12 h) with food and water provided *ad libitum*. All the animals were acclimatized to the laboratory conditions for 7 days before the beginning of the experiments. The control groups: 1- morphine dependent, 2- normal saline (0.9 %) injected and 3- Extract administered. The acute groups: 1- 20 mg nifedipine, 2- 100 mg DEW extract, 3- 300 mg DEW extract. The chronic groups 1: 20 mg nifedipine, 2: 100 mg DEW extract and 3: 300 mg DEW extract. All the groups received subcutaneous injection (s.c) of morphine while naloxone and nifedipine were given intraperitoneally (i.p) except two of the control groups that received the equivalent volume of vehicle (normal saline). All experiments were conducted between 8:00 to16:00 hours.

Morphine Withdrawal Syndrome

A group of 4 rats were chosen randomly for each dose of drugs. Morphine was injected (s.c) twice daily at 8:00 and 16:00 for 5 days as described by Rabbani and colleagues,[3-4] with slight modification. Rats were exposed to escalating doses of morphine from 10 to 60 mg/kg over a period of 5 days. 10 mg/kg, 20 mg/kg, 30 mg/kg, 40 mg/kg and 60 mg/kg were administered on Day 1, 2, 3, 4 and 5 respectively. The withdrawal signs were precipitated 2 hour after the last morphine injection by i.p administration of opioid antagonist, naloxone (5 mg/kg). Immediately after a naloxone challenge, the rats were individually placed in a plexiglas observation box (45 x 30 x 30cm). Animals were observed for withdrawal behaviors, vertical jumps, standings, tremor, eye ptosis, wet dog shaking, teeth chattering, urination and diarrhea for a period of 30 minutes. These parameters were rated based on scoring crriteria adopted from previous studies, with slight modification.[4, 10] A positive jumping response (when a rat jumped ≥ 4 times during observation period) was assigned a score of 4, hyperactivity response (tremor, eye ptosis, wet dog shaking and teeth chattering) was scored as 3, diarrhea was given a score of 2 and urination was assigned a score of 1. However, if the rat jumped < 4 times a score of 1 per jumped was assigned, a maximum total score of 10 and a minimum score of 0 is attainable.

Acute and chronic drug administration

For acute treatment, the DEW extracts (100 mg and 300 mg) and nifedipine 20 mg were administered only once at 1 hour after the final morphine injection. For chronic treatment, the DEW extracts (100 mg and 300 mg) and nifedipine 20 mg were injected 30 minutes prior to each morphine injection for 5 days.

Evaluation of Antioxidant Activity

The antioxidant capacity was determined following the procedure described by Benzie and Strain (1996) using a Ferric Reducing Antioxidant Potential (FRAP) kit. The FRAP colour solution was prepared by adding 625 µL of FRAP reagent A and B into 6.26 mL Assay buffer. The standard calibration curve for $FeCl_2$ was prepared using 6 points calibrations (1000, 500, 250, 125, 62.5 and 31.25 µM). 20 µL of samples (plasma) were pipetted into 96-well plates in duplicate. 75 µL of FRAP colour solution was added unto each sample. The mixture was then incubated at room temperature for 30 minutes in the dark. Absorbance was read at 560 nm. Samples were compared to a ferrous chloride ($FeCl_2$) standard curve and the FRAP values were expressed as Ferrous Equivalent (FE), the concentration of plasma which gives the same absorbance as 1 mmol ferrous ion.

Statistical analysis

The data were expressed as mean ± standard deviation. One way analysis of variance (ANOVA) was carried out using SPSS (version 20) followed by Bonferroni post-hoc test to identify statistically significant difference between and within groups. $p < 0.05$ was set as significant level.

Results and Discussion

Phenolic compounds are known to exert powerful antioxidants properties. These compounds are receiving attention as potential natural antioxidants due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and metal chelators. The date fruit ethanol-water extract (DEW) had total phenolic content of 43.42 mg GAE/g, followed by methanolic extract (DME) 37.82 mg GAE/g, then the aqueous extract (DA) 29.74 mg GAE/g (Figure 1).

Haider and colleagues[24] reported a mean TPC of 4.69 mg GAE/g and 3.57mg GAE/g for Makran and Hillawi date palms respectively. Similarly, according to Abuelgassim[25] the TPC of date palm leaves was 34.63mg GAE/g, thus higher than that of DA (29.74 mg GAE/g) but lower than DME and DEW. Contrarily, our findings showed significantly higher TPC relative to those reported by Adel and colleagues.[26] The authors studied the Deglet-Nour date variety which recorded highest TPC in methanolic extract (0.86 mg GAE/g) and the least in acetone extract. In a different study by Ghiaba and colleagues,[27] TPC of five Algerian date fruits varieties were studied, namely *Degla Baidha*, *Deglet Nour*, Ghars, Tamjhourt and Tafezauine. Highest TPC was observed in Tafezauine extracts (0.23 mg GAE/g), which is also

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had lower values than the ones obtained in this study. However, another study revealed a higher TPC (55.648 mg GAE/g) in methanol extract of Dora date fruit [28]. Similarly, El-Rayes[29] reported that the highest TPC was obtained with ethanol: water extract than other solvents.

Phenolic compounds have been linked to potent antioxidant potential that help the brain in neutralizing oxidative damages, and studies have shown that oxidative stress contributed to the mechanism of opioid dependence.[13, 30-32] In other related studies, phenolic compounds have been shown to block the voltage-dependent calcium channels in rat vascular smooth muscles causing vasodilatation.[21, 33] Chronic administration of opioids usually results in physical dependence as proved by the appearance of withdrawal symptoms after termination of the drug, or when an opioids antagonist is delivered.[34] In this study, chronic morphine treatment resulted in physical dependence which was shown by various withdrawal signs after naloxone injection. In morphine treated rats, naloxone injection induced a full blown behavioral signs of withdrawal (jumping, tremor, eye ptosis, teeth chattering, wet dog shaking, urination and diarrhea). Standing was observed in all the groups, irrespective of administered treatment. The observed standing could be the animal's method of familiarizing with the newly introduced environment (the plexiglas observation box). In the negative control groups, the injection of naloxone did not produce behavioral withdrawal signs as shown in Table 1.

Moreover, administration of *P. dactylifera* extract and nifedipine exhibited increased antioxidant activity in the plasma as seen in the acute and chronic groups. Thus chronic administration of *P. dactylifera* extract and nifedipine showed high antioxidant activity than single or acute administration. These results indicate that *P. dactylifera* extract is an excellent antioxidant and exhibit better potency as compared to calcium channel antagonist like nifedipine.

The acute effects of nifedipine and DEW on various withdrawal signs are illustrated in Table 1 and Figure 2. The acute administration of nifedipine (AC) 20 mg/kg significantly reduced the withdrawal signs by 50 % of jumps, 50 % of urination and 100 % of diarrhea, hence has no ability to reduced hyperactivity sign as shown in Figure 2. However, the acute administration of extract (AE) 100 mg/kg significantly reduced the withdrawal signs to about 75 % of diarrhea, 25 % of urination, however, full blown jumps and hyperactivity signs were sustained. Similarly, acute administration of extract (AEE) 300 mg/kg significantly reduced the withdrawal signs to about 75 % of jumps, 75 % of diarrhea and 50 % of urination, however, animals still exhibited full blown hyperactivity signs (Figure 2).

The chronic effects of nifedipine and DEW on various withdrawal signs are illustrated in Figure 2 and Table 1. The chronic administration of nifedipine (CC) 20 mg/kg significantly reduced the withdrawal signs up to 100 % of jumps and diarrhea, and 75 % of urination and 50 % of hyperactivity signs as shown in the Figure 2. However, chronic administration of extracts (CE) 100 mg/kg significantly reduced the withdrawal signs to about 100 % of jumps, 75% of diarrhea and urination, however only 25 % of hyperactivity signs. Similarly, chronic administration of extract (CEE) 300 mg/kg significantly reduced the withdrawal syndrome to about 75% jumps and diarrhea, 50 % urination, however, only 25% hyperactivity signs (see Figure 2). This result showed significant reduction in the severity of withdrawal syndrome following administration of nifedipine and DEW. The effect was more prominent in chronically administered nifedipine and DEW. However, hyperactivity sign was significantly reduced after chronic administration of nifedipine only.

As illustrated in Figure 2, the jumps exhibited by morphine dependent rats was 100 % in AE and M, while 50 % in AC and 25 % in AEE and CEE with no jumps observed in the groups CC and CE. This confirmed the ability of the treatments to attenuate jumping in the morphine dependent rats upon chronic administration. Hyperactivity, one of the standard withdrawal signs, was only reduced up to 50% in CC (Figure 2), while 100 % hyperactivity was observed in AC, AE, AEE, and M groups. However, CE and CEE exhibited 75 % hyperactivity. Furthermore, nifedipine administration in both acute and chronic effects has the ability to prevent diarrhea in the morphine dependent rats while 25 % of rats in AE, AEE, CE and CEE groups exhibited diarrhea sign. Conversely, group M had 100 % diarrhea. Only 25 % of rats in groups CC and CE passed urine during observation period, while 50 % of rats in AC and AEE, and all the rats in group M passed urine during observation. However, there is statistical significance ($p < 0.001$) in the all chronic treatment of extracts and nifedipine. Similarly, statistical significant differences were recorded between chronic nifedipine and all the three acute treatments $p < 0.05$. However, no statistical difference ($p > 0.05$) was observed in rats that were administered single dose of extracts and nifedipine.

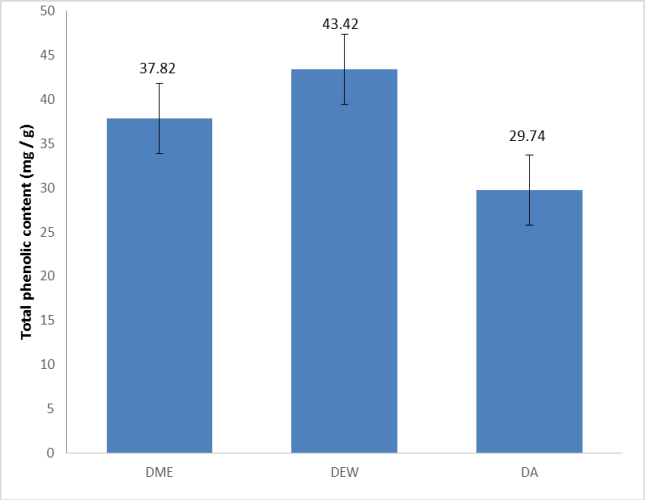

Figure 1: Total phenolic content of methanolic extract (DME), ethanol-water extract (DEW) and aqueous extract (DA).

Table 1: Mean (SD) of withdrawal score with the maximum score of 10 and the minimum score of 0.

Groups	Dose	Mean (SD)
Acute Nifedipine (AC)	20 mg/kg	6.75 (1.26)*
Acute extract (AE)	100 mg/kg	8.25 (0.50) *
Acute extract (AEE)	300 mg/kg	6.50 (1.73)*
Chronic Nifedipine (CC)	20 mg/kg	1.75 (1.50)**
Chronic extract (CE)	100 mg/kg	4.00 (2.58)**
Chronic extract (CEE)	300 mg/kg	4.25 (3.30)**
Morphine	10–60 mg/kg twice.	10.00 (0.00)
Normal saline	Equal vol.	0.00 (0.00)
Extract	300 mg/kg	0.00 (0.00)

* $p < 0.05$ and ** $p < 0.001$.

Table 2: FRAP values of treated and untreated animals.

Samples	Volume (µL)	Ferrous equivalent (mMol/µL)	Antioxidant capacity Mean (SD) (mMol/mL)
AC	20	1.462	73.1 (2.9)
AE	20	1.88	94.0 (1.3)
AEE	20	1.842	92.1 (1.80)
CC	20	1.972	98.6 (0.6)
CE	20	1.994	99.7 (0.6)
CEE	20	2.054	102.7 (1.3)
M	20	0.91	45.5 (0.2)
N	20	2.062	103.1 (0.1)
E	20	2.126	106.3 (0.8)

AC: Acute nifedipine; AE: Acute extract 100 mg/kg; AEE: Acute extract 300mg/kg; CC: Chronic nifedipine; CE: Chronic extract 100 mg/kg/day; CEE: Chronic extract 300 mg/kg/day; M: Morphine dependent; N: Normal saline and E: Extract only.

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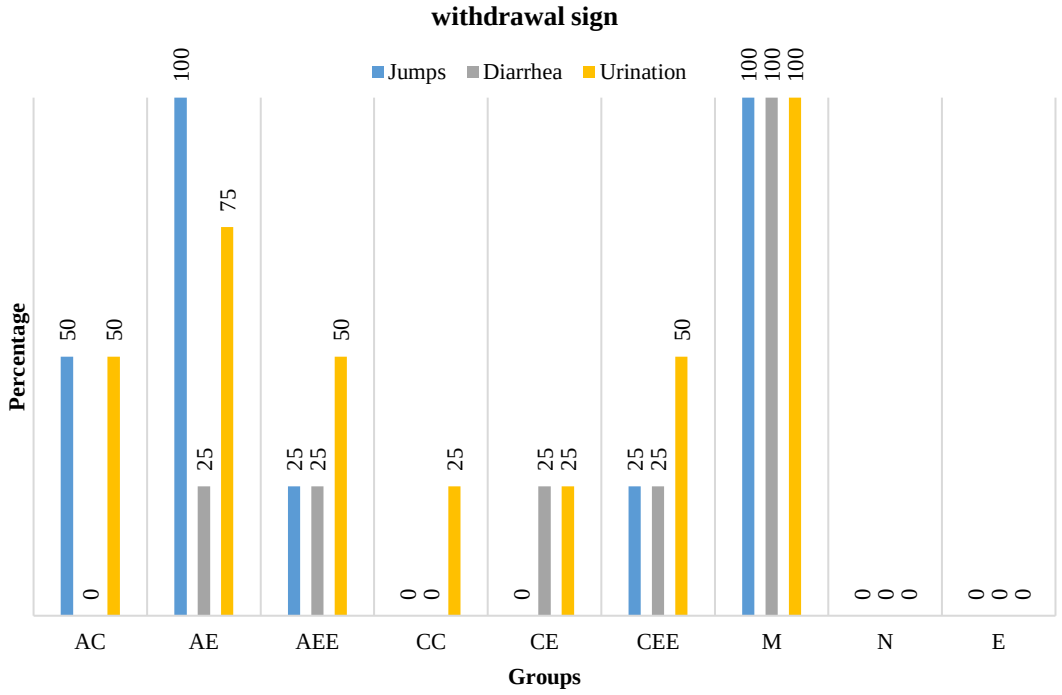

Figure 2: Percentages of the withdrawal sign of the experimental study groups.
AC: Acute nifedipine; AE: Acute extract 100 mg/kg; AEE: Acute extract 300 mg/kg; CC: Chronic nifedipine; CE: Chronic extract 100 mg/kg/day; CEE: Chronic extract 300 mg/kg/day; M: Morphine dependent; N: Normal saline and E: Extract only.

This finding agrees with previous reports.[3, 5, 35-36] Rabbani and colleagues[3] reported significant reduction in withdrawal signs following evaluation of nifedipine (10 and 20 mg/kg) in acute and chronic studies. Moreover, Saboory and colleagues[37] reported significant decreases in the extent and severity of morphine withdrawal symptoms when desmopressin was administered in morphine dependent rats. In a similar study conducted by Schnur and colleagues[38] it was shown that verapamil (20 mg/kg) reversed the naloxone induced withdrawal signs in rats carrying morphine pellets implants. Co-administration of nifedipine and DEW in chronic morphine treatment was more effective in preventing morphine withdrawal syndrome than separate injection of DEW and nifedipine in rats. In this study, DEW co-administered with morphine blocked most of the morphine withdrawal signs. It was also noted that a 4-day treatment with DEW proved to be effective in preventing most of the withdrawal signs of morphine addiction. Extending the use of DEW administration could provide a more effective management of morphine withdrawal syndrome as well as novel non-opioid therapy of opioids addiction. These results provided additional evidence to support the involvement of calcium channels in the adaptive mechanisms responsible for withdrawal signs. In addition to dihydropyridines, phenolic compound in the DEW also seem to be effective in reversing morphine withdrawal signs.

The FRAP values are as shown in the Table 2 and Figure 5. The range of FRAP values among the groups were 45.5 to 106.3 mmol/mL, the group E with 300 mg of date fruit extract (DEW) has the highest value of 106.3 mmol/mL while the group M with escalated morphine treatment has the lowest FRAP value of 45.5 mmol/mL. However, the negative control group (N) has the FRAP value of 103.1 mmol/mL which was higher than that in all the study groups with exception of group E (DEW) which has the highest FRAP value. This showed that morphine administration caused oxidative stress to brain and reduce antioxidant activity as shown in Table 2. All the groups treated with morphine were found to have reduced plasma antioxidant capacity and it was more pronounced in the groups with chronic morphine treatment as indicated in group M. This finding agreed with a study conducted by Guzman and colleagues.[30] The authors reported that the Glutathione (GSH) is the main regulator of the redox balance and contributes to the protection of tissues exposed to oxidizing agent, and showed decrease glutathione levels in both weaned and adult animals and suggested that opioids like morphine unprotected the brain from oxidative stress. However, Sumathi and colleagues[39] stated that the glutathione level in the brain was lowered in morphine treated rats than in the control animals.

Finding of an appropriate drug to treat opioids addiction has been one of the far-reaching goals of scientists. Lack of accomplishment in finding synthetic chemical has inspired the investigators to search natural products for alternatives. A variety of natural products has been tested so far, some with effects and many without significant effects. This study showed that DEW could prevent most of the major signs of the morphine withdrawal syndrome. The exact mechanism of action of the DEW could not be ascertained from these experiments, but we can predict that it has to do with calcium channel antagonist and antioxidant effect. Furthermore, we show that chronic treatment with the DEW reduced the withdrawal syndrome indicative of some reversal of neuronal adaptation that had taken place during chronic morphine treatment.

Conclusion

This study determined the TPC and antioxidant potential of *P. dactylifera* extracts and its effects on calcium channel antagonist in the treatment of withdrawal syndrome in morphine dependent rats. The antioxidant potential of date fruit could be attributed to its phenolic composition. The extract ameliorated cardinal signs of withdrawal syndrome in morphine dependent rats possibly through calcium channel antagonism. Chronic administration of *P. dactylifera* extracts resulted in better reduction in hyperactivity profile relative to acute extract administration. Thus *P. dactylifera* extracts could serve as potential therapy for opioid addiction and prevention of morphine withdrawal syndrome.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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