

Analgesic Property of *Loranthus Acaciae* Studied by Molecular Docking and Biological Assays

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ABSTRACT

Loranthus acaciae (Loranthaceae) is a perennial semiparasitic mistletoe. This study investigates the analgesic, antidepressant and anxiolytic effects of this plant. Different animal models were used. Also, chemical composition of the extract was analyzed using LC-MS. In writhing test, 150 and 300 mg/kg of *L. acaciae* inhibited abdominal cramps by 94.14 % and 94.78 %, respectively compared to 57.95 % inhibition produced by 70 mg/kg indomethacin. In hot-plate but not tail-flick test, 300 mg/kg *L. acaciae* significantly increased latency time. In formalin test, 300 mg/kg *L. acaciae* decreased paw-licking and flinching in early and late phases of formalin test by 68.5% and 83.3%, respectively compared to 39.2% and 56.1% inhibition by indomethacin (50 mg/kg). Naloxone and caffeine but not glibenclamide reversed the analgesic action of this plant in late phase while only caffeine reversed its action in early phase. In forced swimming and elevated plus maze tests, no statistically significant difference was found between *L. acaciae*-treated mice and control group. In open field test, *L. acaciae* decreased the number of lines crossed and rearing behavior. LC-MS analysis of the methanolic leaf extract identified 17 compounds. Loranthin was the major constituent. Based on the results of molecular docking, the activity of *L. acaciae* could be due to the binding of lupeol, campesterol & rhoifolin to μ -opioid receptor. This study indicates that the analgesic action of *L. acaciae* was mediated by interaction with opioid and adenosine receptors. No antidepressant or anxiolytic effects were exerted by *L. acaciae*.

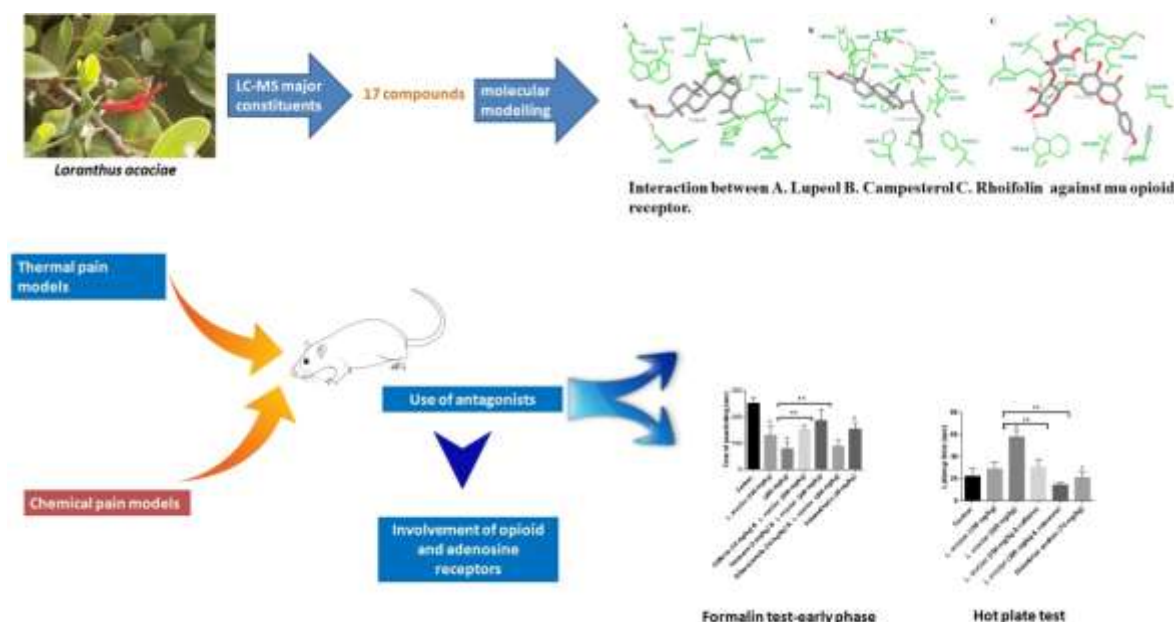
Keywords: *Loranthus acaciae*, analgesic, μ -opioid, adenosine receptor, molecular docking.

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Graphical abstract

INTRODUCTION

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Merskey & Bogduk, 1994). It is estimated that 60% of adults above the age of 65 suffer from chronic pain (Brandt, Beyer, & Stahl, 2012). Chronic pain affects badly the quality of life and causes high cost of treatment as well as loss of

productivity (Sondermann, 2019). Currently available treatments of pain include opioids, nonsteroidal anti-inflammatory drugs (NSAIDs), anticonvulsants and antidepressants (Turk & Melzack, 2011). Opioids may cause many side-effects like constipation, urinary retention, respiratory depression and addiction (Alsalem et al., 2019; Alsalem et al., 2020; Badal, Turfus, Rajnarayanan, Wilson-Clarke, & Sandiford, 2018). NSAIDs

use is limited by their side effects on gastrointestinal and cardiovascular systems while gabapentinoids, antiepileptic drugs, can produce sedation and weight gain (Moran & Szallasi, 2018).

Natural products have earned increasing attention in research and in the development of new drugs (Walker et al., 2017). In traditional medicine they are used as teas, herbal supplements, and formulated products. It is difficult to maintain standardized efficacy, quality, and safety of crude plant extracts and to investigate their mechanisms of action. Therefore, isolation and identification of natural products represent the first step in drug discovery (Wangchuk & Loukas, 2018).

Of 1562 drugs approved between 1981 and 2014, 73% of these drugs were developed from lead compounds derived from natural products (Newman & Cragg, 2016). Therefore, research of natural products was essential for drug discovery of lead compounds for the pharmaceutical industry (Mathur & Hoskins, 2017). Examples of analgesic medicines that were derived from plants are morphine which was isolated from opium (Beutler, 2009), capsaicin from chilli peppers and aspirin obtained from willow tree bark (Abdel-Rahman, 2017).

Loranthus acaciae (syn. *Plicosepalus acacia*, Loranthaceae) is a perennial green semiparasitic mistletoe that directly attaches to another plant via a haustorium (Badr, Shaala, & Youssef, 2013). *L. acaciae* grows in desert where it parasitizes several species of Acacia. Therefore, it is called Acacia strap flower, commonly (Ali-Shtayeh, Jamous, & Abu-Zeitoun, 2014). Also, it may be found on other plants (Qasem, 2009). Recent research has shown that *L. acaciae* possesses several pharmacological activities. The methanolic extract of *L. acaciae* possesses blood glucose-lowering effect and antioxidant effects in diabetic rats (Aldawsari, Hanafy, Labib, & Badr, 2014). Also, it had a gastroprotective action (Abbas, Kandil, Disi, & Jaffal, 2019). Analgesic effect of the two closely related species *L. regularis* and *L. europaeus* was reported (Mothana et al., 2012; Nasrin et al., 2015). Up to our best knowledge, no previous study has investigated this aspect in *L. acaciae*. In addition, anxiolytic and anti-depressant activities were studied.

MATERIALS AND METHODS

Drugs

Glibenclamide (Glibil) from Hikma Pharmaceuticals (Jordan), Diclofenac sodium and clomipramine hydrochloride were brought from Novartis, Switzerland. Diazepam was purchased from Medochemie LTD, Cyprus. Naloxone hydrochloride was brought from Tocris Bioscience (UK). Indomethacin was from DAD, Jordan. Caffeine was from Janssen, Belgium. All drugs were dissolved in sterile normal saline.

Plant collection and extraction

L. acaciae leaves were collected in July 2014 from South Jordan. The plant was authenticated by Prof. Dawud Al-Eisawi. The dried coarsely powdered leaves were macerated in absolute methanol for 3 days. The solvent was evaporated using rotary evaporator at temperature less than 45 °C. The extract was stored at -20 °C until used.

Experimental animals

All procedures were approved by the ethical committee for the use of experimental animals at Al-Ahliyya Amman University (ethical approval number AAU-2/4/2018). Female BALB/c mice were obtained from the animal house at Al-Ahliyya Amman University. Animals were

maintained at 23±2 °C with 12 hr light/dark cycle. Water and food pellets were available *ad libitum*. Mice were adapted to the laboratory for at least 2 hrs before conducting any experiment. At least 8 mice weighing 20-23g per group were used in each experiment.

Pretreatment with antagonists

Mice were treated intraperitoneally (i.p) with either vehicle, glibenclamide (10 mg/kg), or naloxone (5 mg/kg) 30 min before the i.p administration of vehicle, or *L. acaciae* extract (300 mg/kg or 150 mg/kg). The choice of antagonists' dose and time of administration was based on previous studies (Hajhashemi & Amin, 2011; Jaffal, Abbas, Alsalem, & Al-Najjar, 2019). Caffeine (10 mg/kg) was co-administered with *L. acaciae* extract (300 mg/kg or 150 mg/kg) as in (Sawynok, Reid, & Liu, 2013). In all experiments, the response of mice was examined 30 min after the administration of vehicle or *L. acaciae* extract.

Acetic acid-induced writhing test

Acetic acid (1%, 10 ml/kg) was i.p. injected 30 min after vehicle, *L. acaciae* extract or indomethacin (70 mg/kg). The number of abdominal cramps (10 min after acetic acid injection) was counted for 20 min. The percentage inhibition of the extract or standard drug was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{n}^{\circ} \text{ of writhings in control} - \text{n}^{\circ} \text{ in extract-treated mice}}{\text{n}^{\circ} \text{ in control mice}} \times 100\%$$

Paw licking test (formalin test)

Formalin (2.5%, 20 µl) was injected intraplantarly (ipl) to the right hind paw of the mouse. The total time that the animal spent in licking and flinching response was recorded in 2 phases: early phase (0-5 min) and late phase (25-30 min) after formalin injection.

$$\% \text{ inhibition} = \frac{\text{n}^{\circ} \text{ of paw lickings in control} - \text{n}^{\circ} \text{ in extract-treated mice}}{\text{n}^{\circ} \text{ of paw lickings in control}} \times 100\%$$

Hot-plate test

The hot-plate test was used to measure pain reaction latency. Different animals were assigned to different groups. Each mouse was used once in this experiment. The latency time was recorded and included the time between the animal's placement on a hot plate maintained at 55±1° C and the first jump. A cutoff time of 60 sec was used to prevent tissue damage of the mouse (Jaffal et al., 2019).

Tail flick test

The tail flick test was assessed by immersing the tail in water bath at 55±1° C. The time from immersing the tail till producing the first flick was recorded. A cutoff time was considered 10 sec (Jaffal & Abbas, 2019).

Elevated plus maze (EPM) test.

Each closed and open arm of EPM was 10 cm long and 5 cm wide. Each mouse was placed facing one of the closed arms of EPM (50 cm above ground) and was video recorded by camera for 5 min. Entry into an arm was defined as the entry of all four feet of the mouse into that arm. The total time spent in the open and the closed arms was recorded. The percentage of time spent in open arm was calculated as (time spent in open arm/ total time spent in open and closed arms). All animals were tested only once.

Forced swimming test (FST)

Mice were forced to swim individually in 10 cm wide, 25 cm height glass container filled with water (25±1 °C). The

swimming during a 5 min period was recorded in a room with a control of light and noise. The immobility time was recorded for 3 min after 2 min from the beginning of swimming. Each mouse was considered immobile when it stopped struggling or made only the necessary movement to keep it floating with its head above water.

Spontaneous locomotor activity or open-field test (OFT)

Mice were allowed to adapt to the laboratory conditions for about 2 hrs. The ambulatory behavior was assessed in the OFT. The open-field apparatus consisted of a 30 cm x 40 cm, 20 cm high dimensions. The floor was divided into 20 equal squares. The number of rearing (i.e. the number of times the animal stood on the hind paws) and the number of squares crossed by each animal was recorded during a test period of 3 min. The floor of the arena was cleaned between trials with ethanol solution to eliminate the effect of odors left by the previous mouse. The test was carried out at room temperature ($23 \pm 2^\circ\text{C}$) in a room with a control of light and noise.

Liquid chromatography-mass spectrometry (LC-MS) analysis

LC-MS separation was performed using Agilent Zorbax Eclipse XDB-C18 (2.1x150 mm x 3.5 μm) column, the eluent was monitored by Shimadzu LC-MS 8030. The details of the analysis method were specified previously (Jaffal et al., 2019).

Molecular modelling

The following software packages were utilized in this project:

1. ACD/ChemSketch, (www.acdlabs.com) (ACD/Labs, 2019)
2. Autodock 4.2 (Morris et al., 2009)
3. Biovia Discovery Studio visualizer (<http://accelrys.com>) (BIOVIA, 2019)

Molecular docking simulations were conducted to investigate the mechanism of action for plant constituents. The Simulations were performed using the crystal structure of an active μ -opioid receptor downloaded from protein data bank with pdb code: 5C1M (Huang et al., 2015). Compounds found in the plant (Table 1) and 4VO (co-crystal ligand) have been initially prepared by ChemSketch software and then converted to pdb files via Biovia DS visualiser. Gasteiger and Kollman charges were added for small molecules and the protein, respectively, using AutoDockTools. Grid maps were calculated within a grid box of 15, 15 and 15 Å (x, y and z, respectively) using AutoGrid 4 (The Scripps Research Institute, San Diego, CA, USA). Molecular docking simulations of the prepared compounds were performed using AutoDock 4.2 employing Lamarckian genetic algorithm (LGA) for energy optimization and minimization the simulations.

Table 1 Chemical constituents of *L. acaciae* methanolic leaf extract as detected by LC-MS analysis and have been used in the modelling part.

Compound	%
Linamarin	7.9
Catechin	8.3
Epicatechin 3-o- gallate	8.8
Quercetin 3-o-D-glucopyranoside	6.4
Rutin (Quercetin 3-rutinoside)	8.2
Peltatoside (Quercetin-3-O-arabinoglucoside)	9.7
Loranthin	16.9
Lupinine	8.4
Lupeol	3.6
(+) catechin 8-c-rhamnoside	2.7
Campesterol	3.7
Quercetin	4.2
Gallic acid	2.1
Methyl gallate	3.1
Rhoifolin	1.0
Kaempferol	3.0
Apigenin	2.0

Statistical analysis

In all experiments, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was done using GraphPad Prism version 7. $P < 0.05$ was considered significant.

RESULTS

Writhing, hot-plate and tail flick tests

The mean number of writhings in *L. acacia*-treated groups was significantly lower ($p < 0.05$) than in vehicle-treated animals. Mean number of abdominal cramps was 71.11 ± 2.68 , 29.90 ± 1.61 , 4.17 ± 0.79 , 3.71 ± 0.87 for vehicle, 70 mg/kg indomethacin, 150 and 300 mg/kg *L. acacia*, respectively. Therefore, the percentage inhibition of 150 *L. acaciae* methanolic leaf extract was 94.14 % while it

was 94.78 %, for the 300 mg/kg dose compared to 57.95 % caused by the administration of 70 mg/kg indomethacin.

In hot plate test, the highest dose (300 mg/kg) of *L. acaciae* extract increased latency at 30 min significantly. The lower dose of (150 mg/kg) was not statistically different from the control. Both naloxone and caffeine were able to antagonize the action of *L. acaciae* in this test (Figure 1A).

In tail flick test, both doses of *L. acaciae* extract (300 mg/kg and 150 mg/kg) were not effective in increasing the latency time. Indomethacin (40 mg/kg), on the other hand, increased latency time significantly (Figure 1B).

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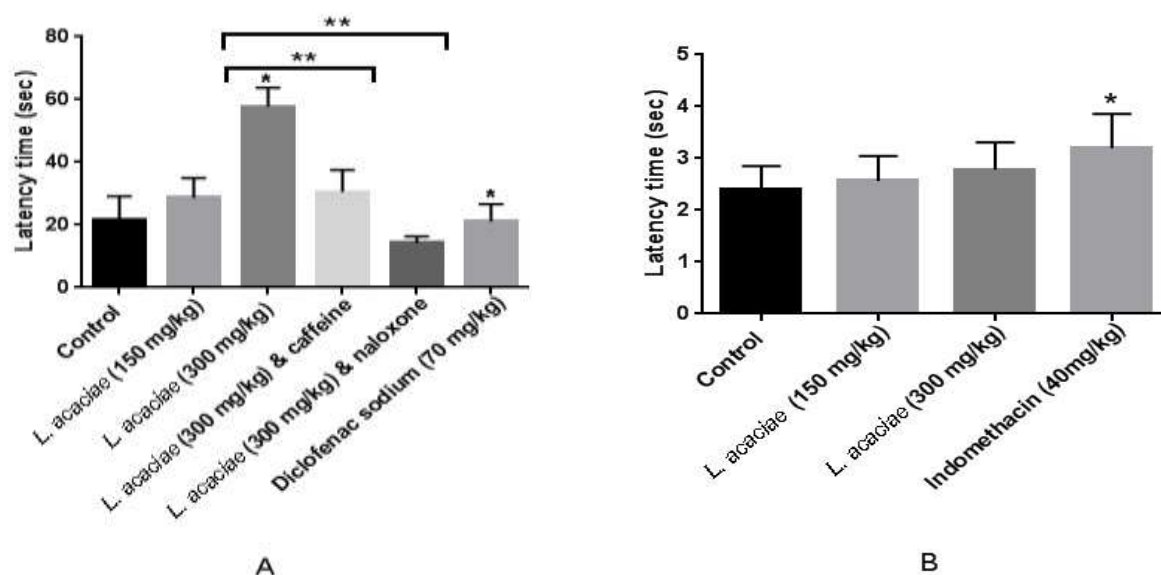


Fig. 1 Results of hot plate test (A) and tail flick test (B).

* Significant difference from the control (p < 0.05).

** P < 0.05 significantly different from *L. acaciae* (300 mg/kg).

Formalin test

In formalin test, *L. acaciae* extracts (150 mg/kg and 300 mg/kg) decreased flinching and paw-licking in early phase of formalin test by 47.9% and 68.5%, respectively compared to 39.2% inhibition caused by indomethacin (50 mg/kg). The percentage inhibition in late phase was 31.1%, 83.3% and 56.1% caused by 150 mg/kg, 300 mg/kg *L. acacia* and indomethacin, respectively. The

opioid receptor antagonist naloxone reversed the analgesic action of this plant in early phase only (Figure 2A) while caffeine, a non-selective adenosine receptor antagonist, reversed the analgesic action of this plant in both early and late phases (Figure 2B). The ATP-dependent potassium channel blocker, glibenclamide, failed to antagonize *L. acaciae* action in both phases of formalin test (Figure 2).

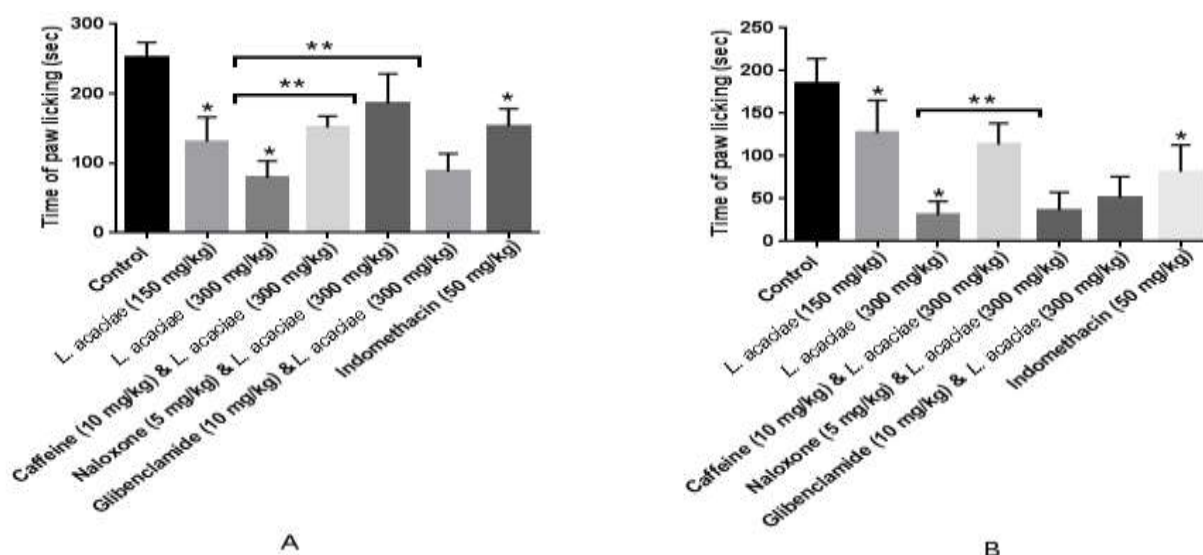


Fig. 2. Results of paw-licking test (formalin test). (A) Early phase (0-5 min after injection) (B) Late phase (25-30 min after injection).

* Significantly different from the control (p < 0.05).

** P < 0.05 significantly different from *L. acaciae* (300 mg/kg).

EPM, FST and OFT tests

In EPM test *L. acaciae* extract had no effect on the time spent in open arm while diazepam increased the time spent in open arm significantly (Table 2). Similar results for diazepam were found in previous studies (Tabari & Tehrani, 2017).

Table 2. Results of EPM, FST and OFT.

Test	EPM	FST	OFT	
			Number of lines crossed	Number of rearing
Control	1.8 \pm 3.91	159.38 \pm 9.09	87.30 \pm 15.27	18.67 \pm 3.39
<i>L. acaciae</i> (150 mg/kg)	0.0 \pm 0.0	171.13 \pm 6.92	17.33* \pm 6.56	3.83* \pm 2.64
<i>L. acaciae</i> (300 mg/kg)	3.86 \pm 4.30	161.13 \pm 12.86	13.83* \pm 7.44	2.33* \pm 1.37
Standard drug #	261.80* \pm 21.38	119.17* \pm 14.57	60.29* \pm 10.78	4.60* \pm 2.12

*Significantly different from the control at $p < 0.05$.

Diazepam (4 mg/kg) in OFT & EPM, clomipramine hydrochloride (40 mg/kg) in FST.

In FST, no statistically significant difference was found between *L. acaciae* extract-treated mice and control group. Clomipramine hydrochloride (standard drug) increased immobility time (Table 2). FST is the most widely used test for assessing antidepressant activity. It is well established that antidepressants like clomipramine reduce immobility time (Mousavi, Fashi, Jahromy, & Rasooli, 2017).

In OFT, both doses of *L. acaciae* as well as the standard drug diazepam decreased significantly the number of lines crossed as well as rearing behavior (Table 2). The results of this study indicate the presence of sedative action of *L. acaciae*. Previous studies reported that low doses of diazepam (e.g 2 mg/kg) produced sedative effect and decreased the number of lines crossed as well as rearing behavior (Fraga et al., 2018).

LC-MS analysis of *L. acaciae* methanolic leaf extract chemical constituents

LC-MS analysis of the extract resulted in the identification of 17 compounds (Table 1). The major compound was the

polyhydroxylated flavanocoumarin loranthin and constituted 16.9% of the extract. The flavonoids quercetin and its glucosides, catechin, epicatechin 3-o-gallate, the cyanogenic glucoside linamarin and the quinolizidine alkaloid lupinine were major compounds in *L. acaciae* extract (Table 1).

Molecular modelling

The co-crystallised ligand (4VO) was successfully re-docked against 5C1M crystal structure with an RMSD of 0.74 Å and -12.04 Kcal/mol free energy of binding. Molecular docking simulations that showed an RMSD values of less than 2.0 Å are believed to have performed effectively (Hevener et al., 2009), Figure 3. Similar parameters were used to dock plant constituents within the active site. The structure performed hydrogen bond interactions with His54 and Asp147 as shown in Figure 4-D. Other amino acids contribute in hydrophobic and VDW interactions.

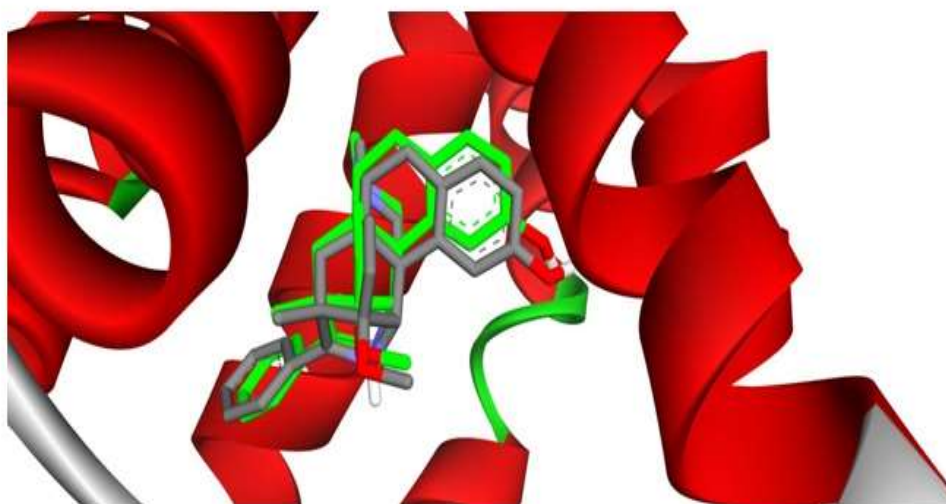


Fig. 3. Flat ribbon representation of μ -opioid receptor (PDB code: 5C1M) crystal structure bound with the co-crystallised ligand (grey) and the re-docked conformation (green).

Plant constituents were successfully docked against μ -opioid receptor, and the results are shown in Table 3. According to the energy results, lupeol, campesterol and rhoifolin showed the lowest binding energies among other compounds, with -12.5, -11.71 and 10.19 Kcal/mol, respectively. The intermolecular interactions against μ -opioid receptor are illustrated in Figure 4, in which lupeol performs one hydrogen bond interaction with His54

while campesterol performs one hydrogen bond with Ile322. On the other hand, rhoifolin performs four hydrogen bonds with His54, Asp147, His297 and Trp318. From previous results, rhoifolin perform similar hydrogen bond interaction with the co-crystallised ligand 4VO by interacting with His54 and Asp147 (Huang et al., 2015).

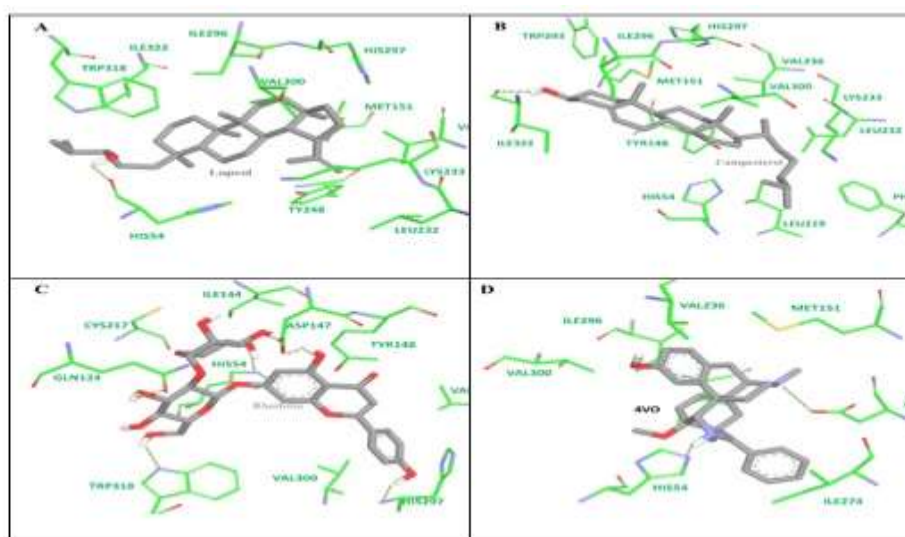


Fig. 4. Stick representation of intermolecular interaction between A. Lupeol B. Campesterol C. Rhoifolin and D. Co-crystallised ligand (4VO) against μ -opioid receptor (pdb code: 5C1M). The hydrogen bond interactions are represented by green-dashed lines. Created by Biovia Discovery Studio visualiser (BIOVIA, 2019).

Table 3. The lowest binding energies for plant constituents against μ -opioid receptor (pdb code: 5C1M).

Compound	Lowest Free Energy of Binding (Kcal/mol)	Compound	Lowest Free Energy of Binding (Kcal/mol)
Epicatechin gallate (ECG)	-9.5	Rhoifolin	-10.19
Gallic acid (GA)	-4.12	Rutin	-9.76
Kaempferol	-7.36	Catechin 3-O-alpha-L-rhamnoside	-9.07
loranthin	-9.34	Catechin	-7.69
Lupeol	-12.5	Campesterol	-11.71
Lupinine	-5.73	Apigenin	-7.58
Methyl gallate	-4.47	Quercetin 3-O-beta-D-glucopyranoside	-8.76
Peltatoside	-9.52	Linamarin	-5.5
quercetin	-7.45	4VO	-12.03

DISCUSSION

Writhing, hot-plate and tail flick tests

In the present study, *L. acaciae* leaf extract exhibited analgesic effects in writhing, formalin and hot-plate tests. Up to our best knowledge, this is the first report of such activity of this plant. Similar analgesic effects were reported for the closely related plant *L. europaeus* in which 400 mg/kg leaf extract produced 59.66% inhibition in writhing test (Nasrin et al., 2015).

In hot plate test, but not tail flick, *L. acaciae* increased latency time significantly. In hot plate test, both caffeine and naloxone antagonized the action of *L. acaciae*. It is well known that hot plate measures supraspinal response while tail flick is considered a reflexive nociceptive test (Gunn, Bobeck, Weber, & Morgan, 2011). This indicates that *L. acaciae* acts at surspinal but not at spinal level. Similarly, the crude extract of *L. regularis* showed dose-dependent analgesic activity in hot plate test (Mothana et al., 2012). In our study, only the high dose (300 mg/kg) but not the lower dose (150 mg/kg) increased latency time in hot plate test. Earlier reports showed that *L. europaeus* extracts increased tail withdrawal latency in tail immersion (tail flick) test (Nasrin et al., 2015).

Formalin test

The effect of *L. acaciae* in paw-licking test was dose-dependent, and the higher dose was more efficient in

inhibiting phase II (83.3% inhibition) than phase I (68.5% inhibition) of formalin test. It is well established that two phases of licking behavior exist in paw-licking (formalin) test. The early or neurogenic phase (phase I) starts immediately after injecting formalin in the foot pad. This early phase is due to the activation of nociceptive sensory neurons by the chemical. Phase II of formalin test is called the inflammatory or late phase because it starts 20-30 min after formalin injection. This phase is due to central sensitization of spinal cord circuits secondary to the inputs that occurred during phase I (Abotsi et al., 2017). In early phase of formalin test, both caffeine and naloxone antagonized the action of *L. acaciae*. In late phase of formalin test, only caffeine was effective. This indicates that at least 2 receptors are involved in the analgesic action of this plant.

EPM, FST and OFT tests

In EPM test *L. acaciae* extract had no effect on the time spent in open arm while diazepam increased the time spent in open arm significantly. Similar results for diazepam were found in previous studies (Tabari & Tehrani, 2017). In FST, no statistically significant difference was found between *L. acaciae* extract-treated mice and control group. Clomipramine hydrochloride (standard drug) increased immobility time. FST is the most widely used test for assessing antidepressant

activity. It is well established that antidepressants like clomipramine reduce immobility time (Mousavi et al., 2017).

In OFT, both doses of *L. acaciae* as well as the standard drug diazepam decreased significantly the number of lines crossed as well as rearing behavior. The results of this study indicate the presence of sedative action of *L. acaciae*. Previous studies reported that low doses of diazepam (e.g 2 mg/kg) produced sedative effect and decreased the number of lines crossed as well as rearing behavior (Fraga et al., 2018).

LC-MS analysis of *L. acaciae* methanolic leaf extract chemical constituents

Similarly, LC-MS analysis of the flower extract of *L. acacia*, analyzed previously, detected the presence of loranthin as a major constituent followed by rutin, peltatoside, catechin, epicatechin 3-O-gallate, lupinine and quercetin (Abbas et al., 2019). However, the cyanogenic glucoside linamarin was found only in leaf extract in the current study. Also, catechin, quercetin, rutin, gallic acid, methyl gallate and loranthin were isolated from *L. acaciae* (Syn. *P. acacia*) using phytochemical methods (Badr et al., 2013) as well as quercetin 3-O- β -D-glucopyranoside, (-) catechin, and catechin 7-O-gallate (Noman et al., 2019).

Molecular modelling

Molecular modeling suggested that 3 compounds may interact with μ -opioid receptor namely: lupeol, campesterol and rhoifolin. This agrees with the results of the in-vivo studies on the mechanism of the analgesic activity of lupeol. Lupeol decreased the duration of acute and tonic pain induced by formaldehyde (de Lima et al., 2013; Nsonde Ntandou, 2017) and increased the latency time on the hot plate test. It also, augmented the intensity and latency time on the paw pressure test and decreased the number of abdominal cramps caused by acetic acid (Nsonde Ntandou, 2017). Lupeol analgesic effect was prevented by naloxone, suggesting that this effect is mediated by activation of opioid receptors (Nsonde Ntandou, 2017). Future studies are needed to investigate the in-vitro and in-vivo interaction of campesterol and rhoifolin with opioid receptors.

CONCLUSION

The results of the present study report for the first time the analgesic effects of *L. acaciae*. The interaction of active constituents with μ -opioid and adenosine receptors is suggested as a possible mechanism of its analgesic action.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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