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THE ANALGESIC AND ANTI-INFLAMMATORY EFFECT OF LAWSONE ISOLATED FROM *LAWSONIA INERMIS*

Tayseer Ali Talab, Narjes Alfuraiji, Ali Esmail Al-Snafi

This study aimed *to assess the analgesic and anti-inflammatory effects of lawsone isolated from henna leaves (Lawsonia inermis).*
Methods*: A total of 120 healthy adult mice (weighing about 25±5 g) were included in this study. Sixty mice out of the total were used to determine LD_{50}, 30 mice to determine the anti-inflammatory test, and the remaining (30 mice) were used for an analgesic test. The hot plate was used to determine the analgesic effect, while the anti-inflammatory effect was determined by the ability of the compound to minimize the inflammation and edema caused by the injection of carrageenan.*
Results*: Lawsone was isolated from Lawsonia inermis. A Stuart SMP10 digital melting point apparatus was used for measuring all melting points. Infrared spectrometer FT-IR 400D was used for measuring/recording IR spectra (KBr) which the frequency of absorption was represented as cm^{-1}. For 1H-NMR spectrum recording, a Bruk-spectrophotometer of 400 MHz was used with internal TMS standard, with deuterated Ś 2.51 ppm for acetone-d6, remained solvent signals as well as 13C.NMR was used. TLC was utilized as adsorbent, UV light, or iodine-completed visualization to verify compounds' purities.*
The LD_{50} of the oral lawsone was 96 mg/kg, and the highest dosage that did not kill any of the experimental animals was 80 mg/kg, which was used to investigate lawsone's analgesic and anti-inflammatory effects.
Lawsone and aspirin possessed an analgesic effect compared to the control group ($p<0.0001$ and $p<0.001$, respectively); however, lawsone induced a potent analgesic effect compared to aspirin ($p<0.1$). In contrast, Lawsone and aspirin exerted an anti-inflammatory effect ($p<0.05$) compared to the control group and were equipotent in carrageenan-induced hind paw edema.
Conclusion: *It is concluded that lawsone possesses analgesic and anti-inflammatory effects, which endorse the practical medical importance of Lawsonia inermis. The latter is widely used traditionally for these purposes own to its cost-effectiveness and safety; however, further studies are required to determine the systemic safety of lawsone*
Keywords: *Lawsone, Lawsonia inermis, henna, analgesic, anti-inflammatory, medicinal plants, phytotherapy, herb, pharmacognosy, pharmacology*

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1. Introduction

Lawsonia inermis Linn (Family: Lythraceae) is a plant often known as henna, predominant in tropical and subtropical regions and has been extensively investigated and used worldwide in traditional medicine and as cosmetic. It has been used as a dye for over 9000 years for its cosmetic value. However, it is used traditionally as abortifacients to treat gonorrhea, intestinal amebiasis, headache, sore throat (decoction), as a blood tonic, hair tonic and treats dandruff and scalp disorders for Jaundice, skin disorders, fever, malaria, conjunctivitis, pimples, and scabies [1]. *Lawsonia inermis* Linn is a native of both Africa and Asia. Furthermore, henna is distributed in Egypt, Africa, Ethiopia, Sudan, Somalia, Niger, Zaire, Burkina Faso, Benin, Gambia, Cote D'Ivoire, Ghana, Togo, South Africa, Guinea-Bissau, Guinea, Sierra Leone, Mali, Senegal, Liberia, Comoros, Nigeria, Seychelles as well as in Asia (Pakistan, Sri Lanka, India). Henna is commonly grown in tropical areas, including North and East Africa, the Arabian Peninsula, the Middle East's southern regions, and South Asia [2, 3].

Lawsonia inermis contained of phenolic, flavonoids, saponins, proteins, carbohydrates, xanthones, fat, resin, and tannins, alkaloids, terpenoids, quinones, coumarins, adding to 2-hydroxy-1,4-naphthoquinone (lawsone, which is the main active component of the henna plant) and several alkaloids, phenolics, naphthoquinone derivatives, and flavonoids were isolated from *Lawsonia inermis's* flower, root, bark, and leaves, all of which have been shown to have active compounds [4, 5].

The pharmacological studies on *Lawsonia inermis* revealed antibacterial activity [6, 7], antifungal [8, 9], antiparasitic [10,11], molluscicidal [12], antioxidant [13, 14], hepatoprotective [15, 16], central nervous [17], analgesic [18, 19], anti-inflammatory [20, 21], antipyretic [18], wound [22, 23] and burn healing [24], immuno-

modulatory [25, 26], anti-urolithiatic [27, 28], antidiabetic and hypolipidemic [29, 30], antiulcer [31], antidiarrhoeal [19] , diuretic [32], anticancer [33, 34] and many other pharmacological effects.

This study was designed to isolate lawsone from henna leaves and investigate its analgesic and anti-inflammatory effects using mice as an experimental model.

2. Planning (Methodology) of the research

The methodology was designed as per Fig. 1 to achieve the research aim.

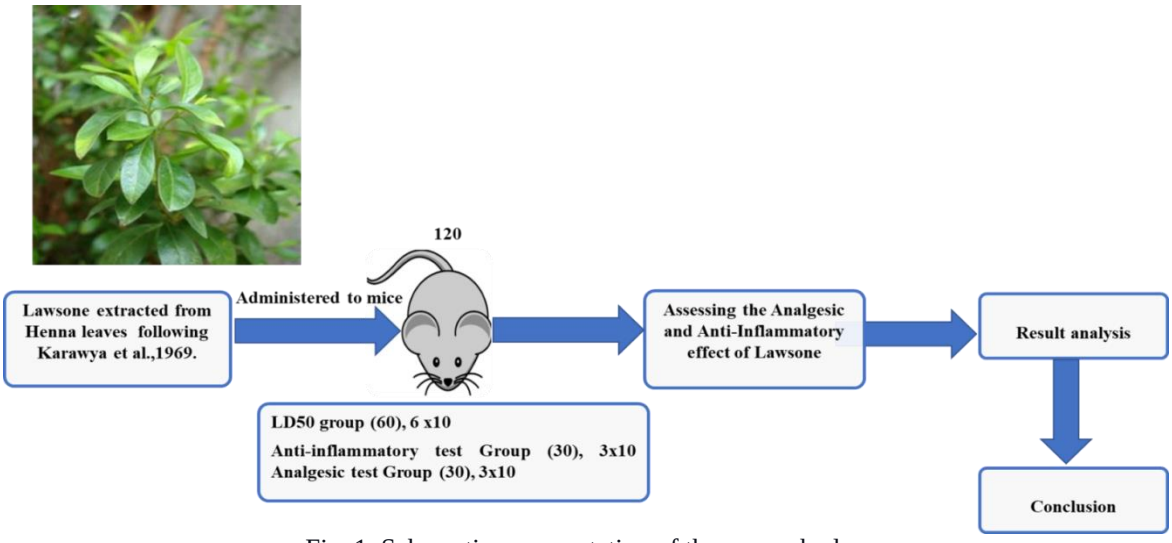

Fig. 1. Schematic representation of the research plan

3. Materials and methods

Chemicals

Lawsone, carrageenan, chloroform, methanol, benzene, ethyl acetate, ammonium hydroxide, and anhydrous sodium carbonate were obtained from Aldrich (Sigma – Aldrich, Germany).

Animals

A total of 120 healthy adult mice (weighing about 25±5 g) were obtained from the laboratory animal house, College of Science, University of Thi-Qar. The study was carried out in the College of Medicine, Thi-Qar university from January to August 2021. Animals were housed in polyacrylic cages and kept under standard laboratory conditions (natural light/dark cycle, RT 22±3°C). Animals were fed a dry rat pellet diet, and tap water was provided *ad libitum*. Thi-Qar university animal ethics committee approved the experimental protocol. Sixty mice were used to determine LD_{50} and 30 mice for the anti-inflammatory test and 30 for an analgesic test. The animal ethics committee supervised by the Institute for the Prevention of Cruelty to Animals and the Research Council reviewed and approved the current study, and Thi-Qar university's animal ethics committee approved the experimental protocol (456/1Q) 24.11.2020. The European Council Directive recommendations (2010/63/EU) on September 22, 2010, amended by Regulation (EU) 2019/1010, on standards to protect animals used experimentally were also followed.

Extraction of lawsone

The leaves of locally grown henna (Southern Iraq, classified by the specialist in the Biology Department-Plant Taxonomy, College of Education, Thi-Qar University) were dried and powdered. One kg of plant leaf powder was extracted for 8 hours with 5 litters of water saturated with anhydrous sodium carbonate. The extract was filtered and was acidified with 1N HCl to pH 3. The lawsone was taken by liter of chloroform, and a separation funnel separated the chloroform and this process. Then the chloroform extract was dried with a rotary evaporator (30 °C), and the lawsone was purified by recrystallization with (methanol: benzene 1:1).

Purification of pigment by chromatography

The residue was shaken in “Solvent A”, a mixture of ethyl acetate, methanol, and ammonium hydroxide (5:15:60). The precipitated lawsone was collected on filter paper and purified using a 50×2.5cm column of silica gel with a mesh size of 60. Before packing, the silica gel was shaken for 1–2 hrs in the solvent “A” in a beaker, and the column was left for 12–15 hrs to equilibrate. A 0.5 g sample in 1ml of solvent (A) was run through the column, and 0.5 ml effluents were collected at a 1 ml/min flow rate. Flash Chromatography apparatus was used to separate Lawsone. The latter was identified using 2-dimension TLC in solvents A compared to known lawsone. [35].

Characterization of isolated Lawsone

A Stuart SMP10 digital melting point apparatus was used for measuring all melting points. Infrared spectrometer FT-IR 400D was used for measuring/recording IR spectra (KBr) which the frequency of absorption was represents as cm^{-1}. For 1H-NMR spectrum recording, a Bruk-spectrophotometer of 400 MHz was used with internal TMS standard, with deuterated Ś 2.51 ppm for aceton-d6 remained solvent signals as well as 13C.NMR was used. TLC was utilized as adsorbent, UV light or iodine-completed visualization for verifying purities of compounds [36].

Determination of LD_{50}

The LD_{50} of lawsone (100, 200, 400, 800, 1600, and 3200 mg/kg) was performed in mice (6 groups,

10 each) as mentioned by Raj et al., 2013 and Al-Ali et al., 2008 [37, 38].

The anti-inflammatory and analgesic tests

The group of the anti-inflammatory test (30 mice) was divided into 3 subgroups. The first one was given normal saline to serve as a control, while the second and third groups were administered with lawsone (80 mg/kg) and aspirin 200 mg/kg BW (both as single oral dose), 60 minutes before the test. A localized inflammation was induced in all the three subgroups by injecting carrageenan (25 μl, 2 % in saline) subcutaneously into the plantar surface of the right hind paw, and the hind paw edema (thickness) induced by carrageenan (mm) was determined half an hour later. The second group was divided and treated in the same manner. The hot plate test was used to determine the analgesic effect of lawsone and aspirin after 60 minutes. The mouse hot plate analgesic assay tests painful stimulation from heat sensitivity. The temperature of the hot plate was maintained at 55°C. Responses such as jumping, the paws' withdrawal, and the paws' licking were observed. The time (latency period) when animals were placed and until responses occurred was recorded by the stopwatch and considered the analgesic period [39, 40].

Statistical methods. The Student t-test was applied to investigate the significance among the groups (SPSS 12.0 software, USA).

4. Results

The UV, FTIR, 1H NMR, and 13C.NMR- spectroscopy techniques were used for the structural characterization. UV max at 273–325 nm for the same compound indicates conjugation (Fig. 2). The peak detected at 1413 and 1471 cm-1indicates of C=C in the IR spectra. The peak at 1616 cm^{-1}indicated the presence of a carbonyl group and a stretching absorption band at 3380 cm^{-1} corresponding to the vibration of the related O–H bond (Fig. 3).

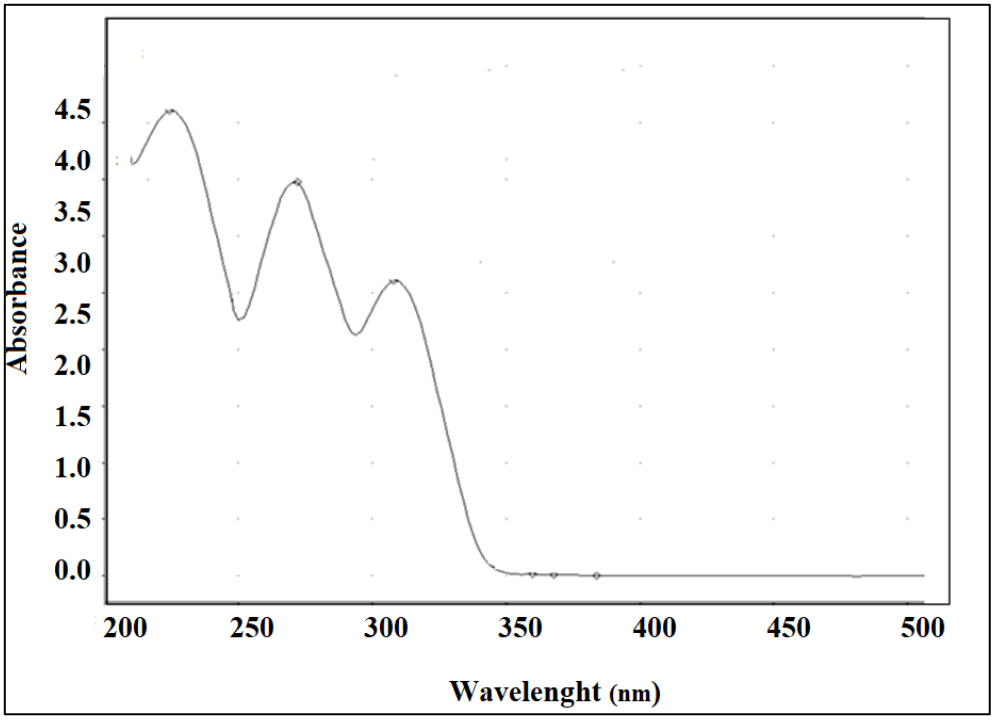

Fig. 2. Shows UV Spectral analysis of the isolated lawsone

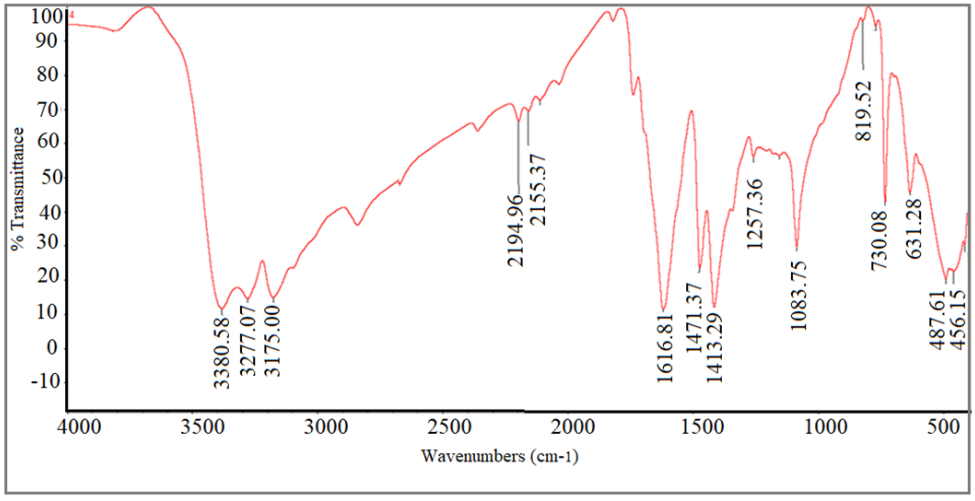

Fig. 3. FT-IR Spectral analysis of isolated lawsone

In the ^{1}H-NMR spectrum (Fig 4), the singlets at 2.04 and 2.84 are refer to the solvent (aceton-d6). The singlet at 6.35 (1H, s) refers to the proton attached to carbon 3 (3C-H). The signal at 7.19-7.57 (3H, 1.3 Hz) for (7C-H) appeared as doublet of triplet with *J*=7.8 was assigned to (7C-H). Also, presence of doublet of triplet 7.70 (td, *J*=7.8, 1.3 Hz) was assigned to (6C-H). The signals at 8.18 (ddd, *J*=7.8, 1.3 Hz) and 8.20 (1H, ddd, 0.5 Hz), corresponding to (5C-H) and (8C-H) respectively. Based on spectral data, the compound was identified as lawsone.

The ^{13}C.NMR spectra (Fig. 5) was also identified a lawsone structure. Moreover, Fig. 6 shows the values of each carbon atoms. This data (UV, FT-IR, ^{1}H.NMR and ^{13}C.NMR) were found to clearly identified and conformed the lawsone structure.

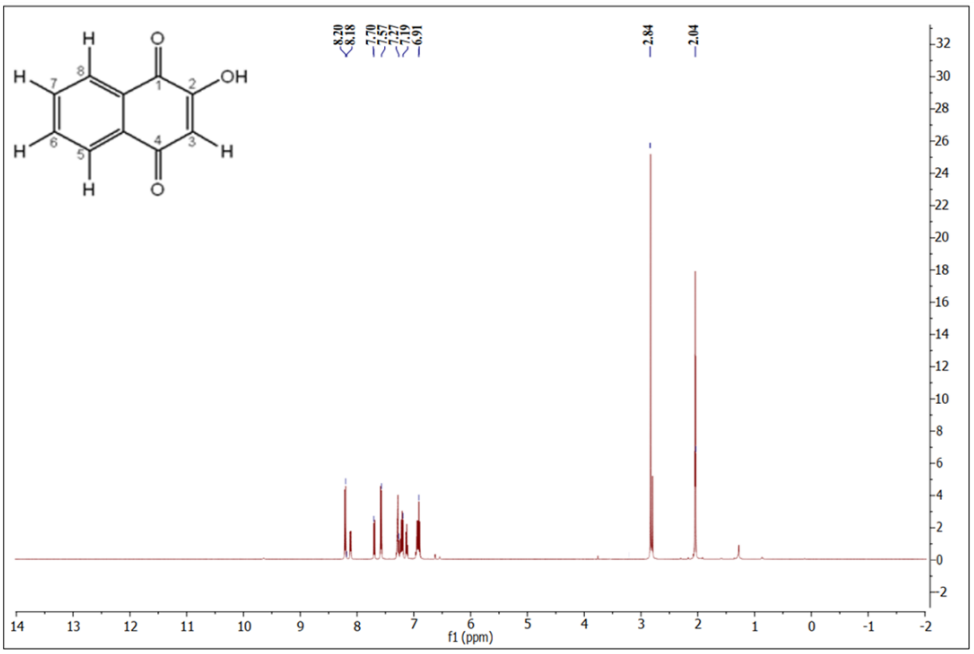

Fig 4.^{1}H.NMR Spectral analysis of isolated lawsone

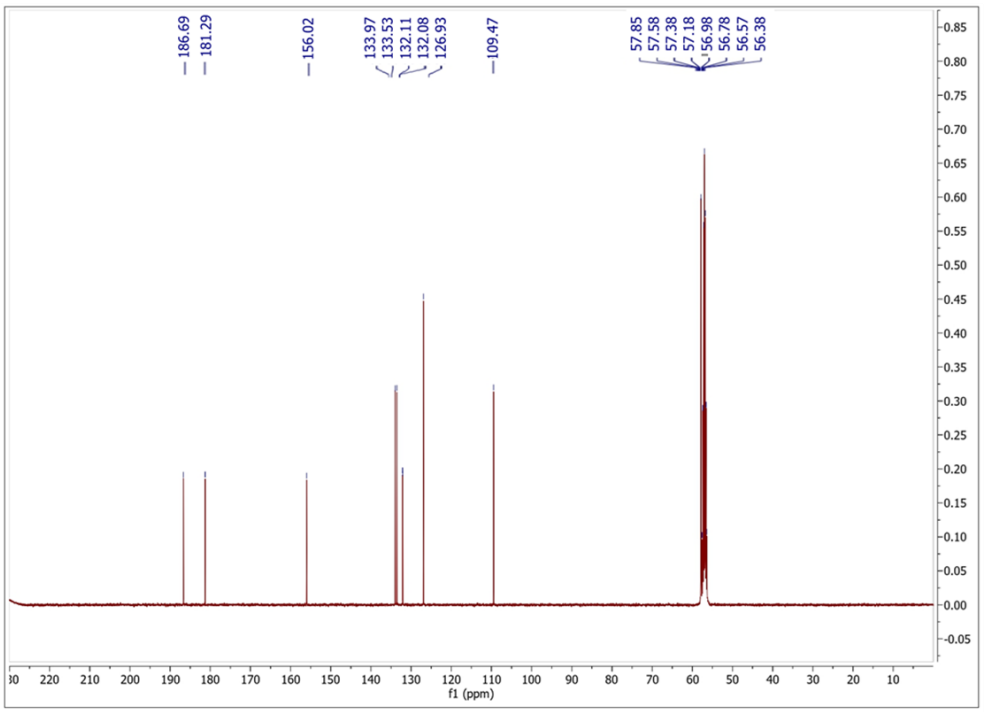

Fig 5.^{13}C.NMR Spectral analysis of isolated lawsone

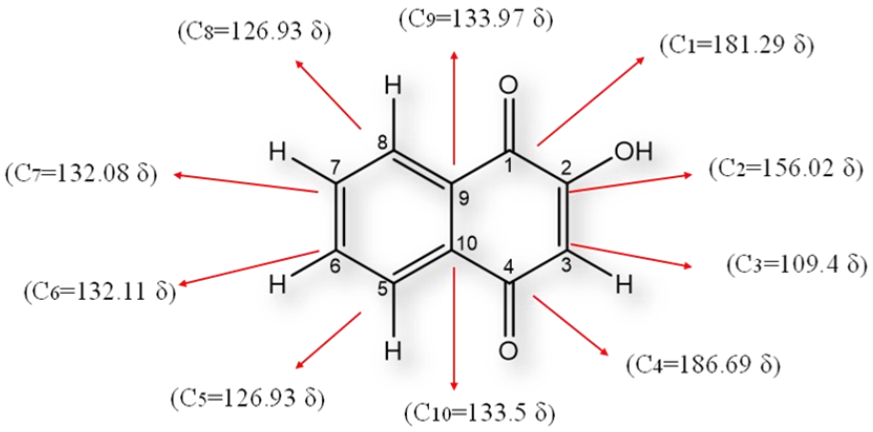

Fig 6.[13]C.NMR Positions for isolated lawsone

Lawsonia inermis leaves contained 1.2 % of lawsone, its melting point was 193 C. Both the extracted and standard lawsone showed Rf of 0.58 on TLC. The mixture of the extracted and standard lawsone gave one spot with the same $R_f$of the standard lawsone on TLC.

The LD_{50} of the oral lawsone in mice was 96 mg/kg. The highest dosage that did not kill any of the experimental animals was 80 mg/kg.

Both lawsone and aspirin possessed an analgesic effect compared to the control group ($p<0.0001$ and $p<0.001$, respectively). However, lawsone induced potent analgesic effect compared with aspirin as determine by the time of analgesic period recorded in hot plate model ($p<0.1$) (Table 1).

Table 1

The analgesic effect of lawsone in comparison with aspirin and control groups

Groups	The period of analgesia	Significancy
Control	6.20±0.26	Lawsone vs Control p<0.0001 Aspirin vs Control p<0.01 Lawsone vs Aspirin p<0.1
Lawsone	23.50±0.57	
Aspirin	9.75±1.70	

On the other hand, both lawsone and aspirin exert anti-inflammatory effect ($p<0.05$) compared with control group. They were equipotent in carrageenan induced hind paw oedema (Table 2).

Table 2

Effect of lawsone on hind paw oedema (thickness) induced by carrageenan (mm) compared with aspirin and control group

Groups	Inhibition of hind paw oedema (thickness) induced by carrageenan (mm)	Significancy
Control	1.53±0.43	Lawsone vs Control p<0.05 Aspirin vs Control p<0.05 Lawsone vs Aspirin Not significant
Lawsone	1.20±0.10	
Aspirin	1.13±0.25	

5. Discussion

Our study revealed that the lawsone content in henna leaves was 1.2 %, slightly higher than some researchers indicated [35, 40]. The melting point of lawsone in our study was 193 ° C. It lies within the melting range of ozone 192-195 ° C. The Rf of the extracted lawsone in this study was 0.58, like that recorded by Karawya et al. (0.57) when using the same solutions [41].

The highest safe dose of lawsone produced an analgesic effect more potent than aspirin, but resulted in a similar anti-inflammatory effect. Many studies showed that *Lawsonia inermis* possessed anti-inflammatory and analgesic effects.

In rats, the raw ethanolic extract of Lawsonia inermis (0.25–2.0 g/kg) had substantial analgesic, anti-inflammatory, and antipyretic effects that were dose dependent. Crude extracts appeared to have less potent analgesic, antipyretic, and anti-inflammatory effects than chloroform and butanol fractions, with butanolic extract (500 mg/kg) shown more effective in the analgesia tests. Methanolic extract of *Lawsonia inermis* also showed peripheral and central analgesic, antipyretic and anti-inflammatory activity in rats [42].

The analgesic, anti-inflammatory, and antipyretic activity of a pure compound isolated from the chloroform extract (2-hydroxy-1,4-naphthoquinone, lawsone) were significant. The anti-inflammatory effect of lawsone (500 mg/kg) was not significantly different from that of the reference drug phenylbutazone (100 mg/kg) [43]. The antiarthritic potential of aqueous leaf extracts (50, 100, 250, 500, 1000, 2000 g/ml) was assessed using the % inhibition of protein denaturation and the membrane lysis method. The aqueous extract showed a dose-dependent antiarthritic effect that is statistically comparable to diclofenac sodium [21, 44].

The anti-inflammatory and analgesic effect of *Lawsonia inermis* leaves with aqueous extract of *Ricinus communis* leaves (as a mixture) were tested in induced knee osteoarthritis in rats (which was induced by intra-articular injection of monosodium iodoacetate). After three days of injection, the results demonstrated a substantial reduction in the injected paws' knee joint width and volume, as well as an improvement in gait analyses. In comparison to the vehicle group, mechanical allodynia analysis after 3 weeks, hotplate latency test after 10 days, spontaneous movements after 7 days, and mechanical

allodynia following two weeks exhibited a substantial analgesic effect. It also shown substantial therapeutic histological alterations in the rats' knees [21, 44].

The methanolic leaves extract appeared to reduce the chemically induced nociceptive pain stimuli significantly ($p<0.01$) [14]. In acetic acid and heat-induced pain models, methanol, petroleum ether, and ethyl acetate extracts of Lawsonia inermis leaves (250 and 500 mg/kg, IP) had a substantial dose-dependent analgesic effect (p 0.05-0.001) [19]. The tail immersion and hot plate techniques were used to investigate the synergistic analgesic effects of chloroform extracts of Lawsonia inermis leaves and roots tubers and Chlorophytum borivilianum in mice. At a dose of 200 mg/kg BW, chloroform extracts of both plants exhibited considerable analgesic activity, and the combination of both extracts produced higher analgesic activity than each extract alone [20].

In mice with acetic acid-induced writhing, the ethanolic extracts showed no substantial inhibition ((28.45 %, p 0.3) of the writhing reflex at a dose of 500 mg/kg compared to diclofenac sodium that showed inhibition of 82.7 %at a 25 mg/kg BW [24, 44].

The anti-inflammatory effect of lawsone could be attributed to its inhibitory effect on cyclooxygenases and subsequent inhibition of prostaglandins synthesis [40]. Furthermore, in studying 5- lipoxygenase (5-LOX) inhibition, the methanolic extract exhibited better 5-LOX inhibition than chloroform and hexane extracts of henna seeds, with an IC50 value of 51 0.23 mg/l [45].

Study limitations. Following the Guidelines of laboratory animals use and regulations of Thi-Qar University, it was not possible to test more laboratory animals in accordance with The European Council Directive recommendations (2010/63/EU), which is based on the principle of Three Rs, replace, reduce, and refine the use of animals used in scientific research.The authors have to conduct their study using a sample size of 120 mice. Sample size could be increased in future studies to have better insight into the efficacy and safety of lawsone. However, the current study is required to suggest further studies that could help in heightening the medical importance of lawsone.

Prospects for further research. Further studies are required, including conducting a well-controlled study with a larger sample size to determine the therapeutic effect of lawsone in vivo. Also, another study is required to determine the teratogenicity of lawsone before introducing lawsone into clinical trials as analgesic and anti-inflammatory**.**

6. Conclusion

Lawsonia inermis leaves contained 1.2 % of lawsone. The LD_{50} of the oral lawsone in rats was 96 mg/kg. An 80 mg/kg was the highest dosage that did not kill any experimental animal. Both lawsone and aspirin possessed analgesic effect compared with control group ($P<0.0001$ and $P<0.001$ respectively). However, lawsone induced potent analgesic effect compared with aspirin ($p<0.1$). On the other hand, both lawsone and aspirin exert anti-inflammatory effect ($p<0.05$) compared with control group. They were equipotent in carrageenan induced hind paw oedema. Accordingly, lawsone possesses potent analgesic and anti-inflammatory effects, which endorse the practical medical importance of *Lawsonia inermis as analgesic and anti-inflammatory medication.*

Conflicts of interest

The authors declare that they have no conflicts of interest.

Financing

The study was performed without financial support.

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