Preparation of *Citrullus colocynthis* Seed Extract and Assessment of Cyto-toxicity, Anti-microbial, Anti-inflammatory, and Analgesic Activities

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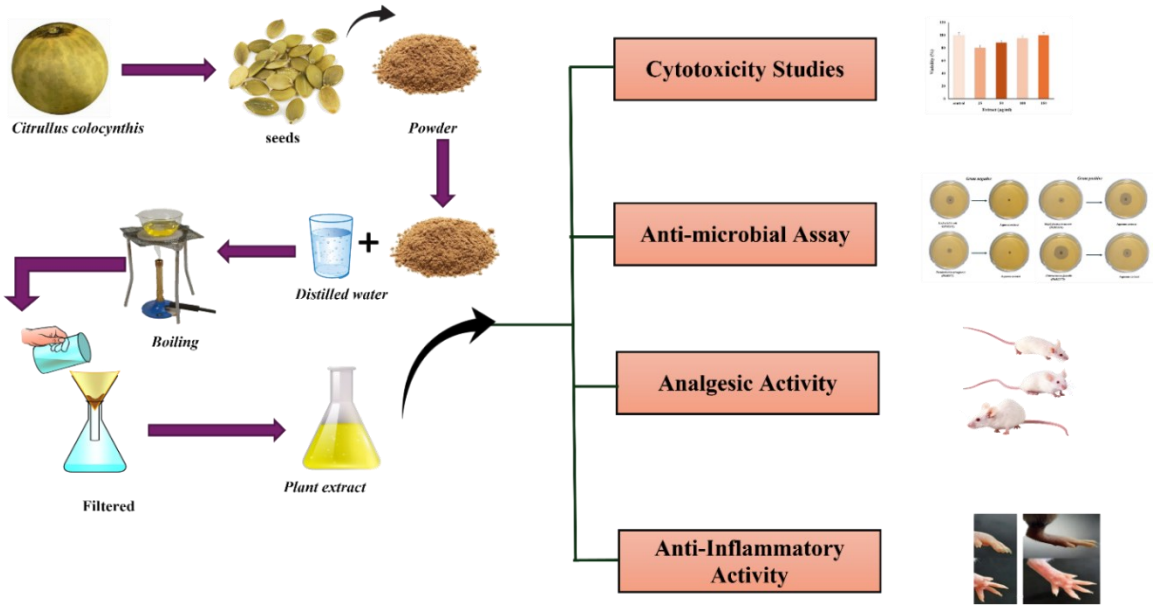
GRAPHICAL ABSTRACT



Preparation of *Citrullus colocynthis* Seed Extract and Assessment of Cyto-toxicity, Anti-microbial, Anti-inflammatory, and Analgesic Activities

Zihan Zhu,[a] Jie Zhang,[b,*] and Huan Meng [c,*]

One of the most common health issues that affect individuals is pain, which can manifest in a variety of ways. Opioids and nonsteroidal anti-inflammatory medications (NSAIDs) are frequently prescribed for pain relief; however, their prolonged use can result in substantial adverse effects on various bodily systems. As a result, it is important to identify alternative medications that are both more effective and secure. The health benefits of *Citrullus colocynthis* herb, rich in beneficial elements, are well documented. The effects of an aqueous seed extract of *C. colocynthis* were examined in this study. The B16F10 melanoma cell line was employed to evaluate the extract's cytotoxicity. The authors implemented antimicrobial investigations employing four bacterial strains. The anti-inflammatory and analgesic activities were assessed using Swiss albino mice and Wistar albino rats, respectively. The cytotoxicity analysis demonstrated that the *C. colocynthis* extract did not manifest any significant cytotoxic effects on the B16F10 melanoma cell line. The extract exhibited efficacious activity against the Gram-positive bacteria that were the subject of antimicrobial investigations. Pain inhibition effects exceeded 70%, with the analgesic activity. Consequently, the *C. colocynthis* extract exhibits potential as an analgesic and antimicrobial agent, necessitating additional research and development work.

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Keywords: Citrullus colocynthis; Cytotoxicity; Anti-microbial; Analgesic; Anti-inflammatory

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INTRODUCTION

The most common human condition known to science is pain in all its forms. One in five individuals globally experiences chronic pain, one in ten adults receives a diagnosis each year according to the International Association for the Study of Pain (IASP 2012), and roughly one in nine young adults deal with chronic pain (Murray *et al.* 2022). Depending on the nature of pain and the variety of research done, children experience pain anywhere from 11% to 38% of the time (Sakurai *et al* 2015). This information emphasizes how important pain is as a major health concern worldwide.

One of the main signs of inflammation is pain, which also manifests as redness, warmth, swelling, and decreased function, all of which together define a typical inflammatory response. Though inflammation is essential to the body's natural equilibrium processes, its effects can occasionally go well beyond preserving equilibrium, as it can

lower pain thresholds and increase sensitivity to pain (Clinkard *et al* 2024; Zhang *et al* 2023).

Medications, such as opioid pain relievers and non-steroidal anti-inflammatory medications (NSAIDs), are frequently used to treat pain. Although these drugs greatly help with the management of pain, some people may experience severe negative effects from them. High blood sugar, gastrointestinal tract irritation, heart problems, liver toxicity, and elevated toxicity risks, especially in older persons, are common adverse responses (Schofield *et al* 2019). Despite extensive research on conventional pain management strategies, such as the use of NSAIDs and opioids, significant challenges persist, including adverse side effects, risks of dependency, and limited efficacy in certain populations. (Bennett *et al* 2024).

Exploring natural substitutes has therefore proved to be a wise course of action. Furthermore, while natural substitutes such as *Citrullus colocynthis* have demonstrated promising pharmacological properties, including analgesic, anti-inflammatory, and antioxidant effects, comprehensive evaluations of its bioactive components in advanced experimental models remain incomplete (Rao and Poonia 2023). It is a desert plant that has great therapeutic potential due to its abundance of bioactive components, including glycosides, fatty acids, alkaloids, flavonoids (Kim *et al* 2022), and essential oils. It is well known that this plant strengthens immunity. The pulp of *C. colocynthis* fruit has long been used to treat digestive disorders, such as dyspepsia, and parasite infections. The seed powder of *C. colocynthis* is a versatile ingredient with medical and culinary applications (Li *et al* 2022). Its antibacterial, analgesic, anti-inflammatory, antioxidant, and fat-binding qualities are demonstrated by its use as an emulsifier, flavoring agent, and fat binder (Kamran *et al* 2018). Specifically, there is a lack of robust evidence regarding its mechanism of action in alleviating pain and inflammation and its comparative efficacy against standard pharmaceutical agents. Additionally, its potential applications in specific formulations or delivery systems to enhance bioavailability and therapeutic effects remain underexplored.

For the first time, this study investigates the pharmacological efficacy of *C. colocynthis* using advanced *in vivo* experimental models, including the hot plate test and the carrageenan-induced rat paw edema assay. The study provides detailed insights into the plant's mechanism of action, focusing on its analgesic and anti-inflammatory effects. Furthermore, the study explores antibacterial properties highlighting their potential for medical and culinary applications. These findings represent a novel contribution to understanding the therapeutic potential of *C. colocynthis* and its practical applications

EXPERIMENTAL

Materials

Foetal bovine serum (FBS), sodium pyruvate, glutamine, and dimethyl sulfoxide (DMSO) was sourced from Sigma Aldrich, based in Shanghai.

Preparation of Plant Extract

C. colocynthis fruits were crushed to obtain seeds for this study. Approximately 5 g of seeds were finely ground into a powder and mixed with 100 mL of distilled water. The mixture was then heated to a boil and allowed to simmer for 10 to 15 min. Then it was set

aside for 2 h. Subsequently, the purified extract was separated from the larger particles using a process of triple filtration.

Cytotoxicity Studies

Cell culture

A 75-cm² flask containing 10% FBS, 1 mM sodium pyruvate, 1% non-essential amino acids, 2 mM L-glutamine, and antibiotics was used to cultivate the B16F10 mouse melanoma cells. The temperature of these cultures was kept at 37 °C in an environment with 5% CO_2.

MTT assay

An MTT assay was used to evaluate the effect of *C. colocynthis* extracts on B16F10 melanoma cells. To perform the experiment, 100 µL of growth media each well in a 96-well plate was used to plate 3×10^3 cells/mL and incubated at 37 °C for 48 h. After incubation, cells were added with different doses of the extract (25 to 150 µg/mL) and allowed to incubate for a further 24 h at 37 °C. Each well was filled with 8 µL of MTT solution and incubated at 37 °C for 4 h. After solubilizing the cells with 100 µL of dimethyl sulfoxide (DMSO), absorbance was measured at 570 nm using an automated microplate reader. There was a direct correlation between the absorbance level and the quantity of viable cells.

Live and dead cell assay by confocal laser scanning microscopy (CLSM) using fluorescein diacetate and propidium iodide staining

The viability of the cells was evaluated qualitatively using confocal microscopy following labelling with propidium iodide (PI) and fluorescein diacetate (FD). Trypsinization was followed by resuspension of the cells in Complete Culture Medium at a density of 10×10^4 cells/mL. After that, a 2 mL aliquot of the cell suspension was put on a cell culture dish and left there for the entire night at 37 °C with 5% CO_2. The following day, the culture media was taken out and replaced with 100 µg/mL of plant extract. After the media were evacuated after a 24 h incubation, 600 µL of FD (10 µg/mL in 0.2% DMSO/PBS) and 600 µL of PI (5 µg/mL in PBS) were added to the cells. After gently mixing the cells, they were incubated for 10 min in the dark. The cells were then twice washed with 1 mL of PBS, and each plate was filled with 1 mL of imaging media before being imaged using CLSM.

Anti-microbial Assay

The Gram-negative microorganisms *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) and the Gram-positive organisms *Staphylococcus aureus (S. aureus)* and *Enterococcus faecalis (E. faecalis)* were used to test the extract's antibacterial activity. Individual colonies from a 24 h culture were suspended in a saline solution on agar plates. Disc diffusion technique was used to evaluate antimicrobial potential of the extracts. Suspensions were evenly distributed across Mueller-Hinton agar (MHA) plates, each holding 25 mL of the medium, for every bacterial strain. Approximately 5 mg of dry extracts were added to each of the individual discs, with a control disc included for comparison. In this study, ampicillin and DMSO were used as positive and negative control, respectively.

Experimental Section

Animals

In this study, 60 4- to 6-month-old Wistar albino rats weighing 150 to 200 g, and 60 male and female Swiss albino mice weighing between 22 to 35 g underwent a 14-day acclimatisation period under standardised housing conditions. Both the mice and rats were housed in cages kept at room temperature that had a 12 h light-dark cycle and provided with a regular pellet meal and unlimited access to water. The present study's methodology and procedures were conducted in strict accordance with the established recommendations for the care and use of laboratory animals (Guide for the Care and Use of Laboratory Animals 2024).

Grouping and dosing

There were five different groups of mice, each consisting of six animals. Group I was given distilled water as negative control. The extract was administered to Groups II, III, and IV at doses of 100, 200, and 400 mg/kg, respectively. These dosages were chosen based on prior studies on acute toxicity (Mergia *et al* 2016). Morphine, the common painkiller, was given to Group V at a dose of 10 mg/kg. The gastric gavage technique was utilised to administer these drugs orally.

Analgesic Activity

Hot plate method

Every mouse was placed on a heated plate with a precisely regulated temperature of 55 ± 1°C. For every mouse observed, the time in seconds between when it was put on the hot plate and when it began to show signs of discomfort (*e.g.*, kicking, jumping, licking, or covering its rear limbs) was carefully noted. The time limit was set at 15 s to prevent the animals' paws from being burned. Before the *C. colocynthis* extract was applied and 30, 60, 90, and 120 min afterward, the times at which the animals reacted to the pain were meticulously recorded. This process made it possible to thoroughly assess how well the extract reduced pain over time and at different dosages. An analysis was conducted to determine how long it took the treated groups to react more slowly than the control group's baseline. Using a preset method, the percentage of the maximal potential antinociceptive effect was evaluated to assess the efficacy of the treatment (Abbas (2013).

$$\text{Max Analgesia} = \frac{\text{Reaction time for the test-Reaction time for negative control x 100}}{\text{15Sec – Reaction time for negative control}} \quad (1)$$

Anti-Inflammatory Activity

Anti-inflammatory properties were studied using rat models and the carrageenan-induced paw swelling approach. The male Albino Wistar rats used in this experiment weighed between 150 and 200 g, with 6 animals per experimental group and these rats were given unlimited access to water but fasted for 24 h prior to test. An hour prior to the test, 50 µL of a 1% carrageenan in saline solution were prepared and injected under the rats' rear paws. The *C. colocynthis* extract was combined with indomethacin in a solution that was combined with a 0.9% w/v saline solution and polysorbate 80 (Tween 80). The final concentration of polysorbate 80 was maintained below 5% to avoid any solvent-induced side effects. Intraperitoneal injections of indomethacin (5, 10, and 15 mg/kg) and

the extract (50, 100, and 150 mg/kg) were used. These shots were administered 1 h before the carrageenan was applied. After the carrageenan injection, paw volumes were measured using a plethysmometer both right away and for up to 4 h afterward. The amount of the induced swelling was ascertained by dividing the total volumes of the hind paws before and after the application of carrageenan. Ratios < 1.5 after treatment demonstrated a marked decrease in edema, demonstrating the strong anti-inflammatory effects of the drugs given (Chi and Jun 1990).

The present animal research was carried out in line with the principles and standards outlined in the institutional Ethical Committee of Animal Experimentation (Approval number: 2022-07-05). All the animal experiments were performed according to ethical committee guidelines of Shanghai Huashan Hospital, Shanghai.

Statistical Analysis

The analysis was conducted using statistical package for social science (SPSS) version 25 and Graph pad prism version 8.1. One way analysis of variance (ANOVA) was used to analyze the data followed by Tukey *post hoc* test to determine statistical significance. All the data were expressed as mean ± standard error of the mean (SEM). P values ≤ 0.05 were taken as statistically significant

RESULTS AND DISCUSSION

Assessment of Cytotoxicity

The effect of the *C. colocynthis* extract on tumour cell line survival was also examined. Specifically, the MTT assay was used to investigate the link between the viability of B16F10 melanoma cells and concentration of the extract. Different concentrations of *C. colocynthis* aqueous extract (25 to 150 µg/mL) were applied to B16-F10 cells for 24 h at 37 °C. The concentration range was chosen by prior research that examined comparable experimental configurations utilizing water extracts and fixed oils derived from *C. coccineum* (Rosa *et al.* 2012). The extract did not significantly harm the melanoma cells, even at higher doses (150 µg/mL), as indicated in Fig. 1. The present results contrast with those of Maisetta *et al.* (2019, who demonstrated that the extracts of Greek *Cytinus* exhibited cytotoxic activity against a variety of cancer cell lines.

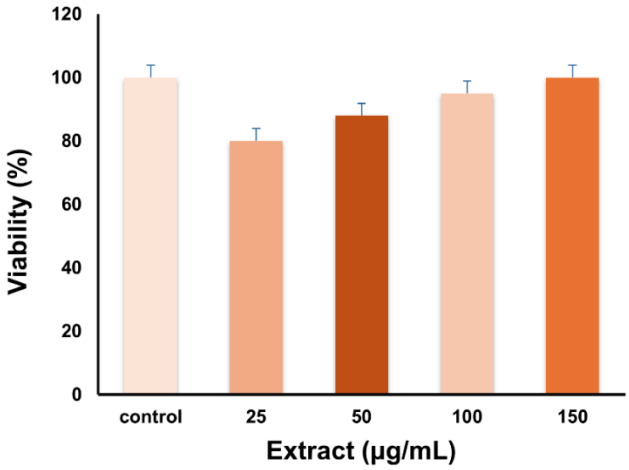

Fig. 1. Viability (expressed as % of the control) induced by 24 h incubation with *C. colocynthis* aqueous extracts in melanoma B16F10 cells. Data are the means ± standard deviations of three independent experiments involving triplicate analyses for each sample.

Live/Dead Staining

Both untreated B16F10 cells and those treated with a concentration of 100 µg/mL of *C. colocynthis* plant extract were subjected to the Live/Dead cell test. As shown in Fig. 2A and 2B, treatment with the extract caused a significant amount of cell death in comparison to the untreated cells. Significant morphological alterations caused by the plant extract resulted in diminished confluency and widespread propidium iodide (PI) staining. These results show that *C. colocynthis* is very hazardous to B16F10 cell lines. Using images from CLSM, the reduced cell viability and enhanced cytotoxicity were verified.

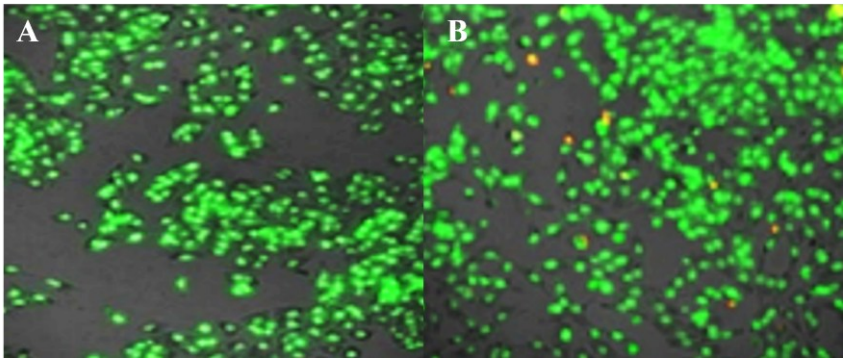

Fig. 2. Live/Dead assay of B16F10 cells (A) untreated and (B) treated with plant extract

Assessment of Anti-Microbial Activity

Table 1 provides a comprehensive overview of *C. colocynthis* extract's antibacterial activities. It is worth mentioning that all the Gram-positive bacteria that were studied were effectively targeted by the *C. colocynthis* extract. More specifically, for every disc treated with 0.5 mg of the extract, inhibitory zones surpassed 10 mm. Research says that the inhibition zones bigger than 9 mm show antibacterial activity and smaller zones suggest ineffectiveness (Olmedo *et al* 2024).

Table 1. Anti-microbial Activity of *C. colocynthis* Extract Using Disc Diffusion Technique

Bacterial Strains	Aqueous Extract 0.5 mg/disc (mm)	Antibiotic Control
Gram Negative		
***Escherichia coli* (DSM1103)**	0.0 ± 0.0	AMP 16.7 ± 0.5
***Pseudomonas aeruginosa* (DSM117)**	0.0 ± 0.0	AMP 12.5 ± 0.7
Gram Positive		
***Staphylococcus aureus* (DSM1104)**	10.0 ± 1.6	AMP 1.2 ± 1.05
***Enterococcus faecalis* (DSM2570)**	8.0 ± 3.1	AMP 22.1 ± 0.5

Under the same experimental conditions, the *C. colocynthis* extract demonstrated a wider range of action but was not as powerful as the conventional antibiotic ampicillin. It is worth mentioning that ampicillin had no effect on MRSA *S. aureus*. Additionally, the *C. colocynthis* aqueous extract showed efficacy against bacteria that are Gram-negative as well (Table 1).

Evaluation of Analgesic Activity

Table 2 illustrates that the CC 150 and CC 200 dose groups significantly reduced pain when compared to the control and CC 100 groups. The MO10 group showed a similar pattern of extended pain response time. Furthermore, during the study, the CC 200 group's effectiveness at alleviating pain was comparable to that of the MO10 group. Surprisingly, the maximum analgesic response was measured after 120 min, and the CC 150, CC 200, and MO 10 groups had pain inhibition percentages of 74%, 85%, and 80%, respectively. CC 200 consistently exceeded DW, CC 100, and CC 150 groups in terms of pain inhibition percentage for the course of the trial, outperforming all tested extract doses. However, CC 200 demonstrated a lower pain inhibition % than MO 10 until the 90-min mark, at which point its effectiveness exceeded that of the MO 10 group. The peak analgesic effects of all experimental groups were observed at 120 minutes in the hot plate test. The duration of this effect may be attributed to the drug's time to penetrate the central compartment and reach the target site (Gordon *et al* 2016). CC100 did not exhibit any significant effects among the tested groups. However, CC150 and CC200 exhibited significant analgesic activity, with statistically significant results ($p < 0.05$ at 30 minutes and $p < 0.001$ at 120 minutes) in comparison to the control. The pharmacological effects of the extracts were further confirmed by the prolonged latency.

Table 2. Effect of Aqueous Seed Extract of *C. colocynthis* on Hot Plate Test in Mice

Group	0	30 Min	%	60 Min	%	90 Min	%	120 Min	%
DW	4.6 ± 0.45	4.8 ± 0.70	-	4.5 ± 0.3	-	4.8 ± 0.6	-	5.2 ± 0.2	-
MO	3.8 ± 0.50	7.0 ± 0.35	29%	7.6 ± 0.5	41%	8.6 ± 0.8	45%	14 ± 0.8	80%
CC-100	3.4 ± 0.49	3.8 ± 0.5	1.7%	4.2 ± 0.51	8.4%	5.0 ± 0.56	18%	6.8 ± 1.1	30%
CC-150	4.2 ± 0.52	6.2 ± 0.34	18%	8.0 ±0.56	36%	8.0 ± 1.5	36%	14 ± 0.6	74%
CC-200	5.2 ± 0.52	7.0 ± 0.50	18%	8.0 ± 0.56	30%	10 ± 1.15	50%	12 ± 0.5	85%

Assessment of Anti-Inflammatory Activity

Table 3 shows the results of the carrageenan-induced paw swelling test with the extract and indomethacin. The carrageenan-injected paws reached their maximum swelling 3 h after the injection, while the control group's average volumetric increase was about 100%. According to statistical analyses, different concentrations of extract (50, 100, and 150 mg/kg) considerably reduced edema compared to the control group. The edema was reduced to 30.8% 3 h after the carrageenan injection in different doses of *C. colocynthis* extract. The results showed that the extract at 150 mg/kg was more effective than indomethacin at 15 mg/kg in reducing edema. There was no significant difference in the effectiveness of edema reduction between the 150 and 100 mg/kg extract doses ($P > 0.05$). The research discovered that indomethacin at 5 mg/kg and the extract at 50 mg/kg both showed similar effectiveness in reducing rat paw swelling brought on by carrageenan. In contrast to the reference medication, the extract exhibited substantial anti-inflammatory properties.

As evidenced by decreased paw edema and higher percent edema protection in carrageenan-induced paw edema model, CC 100 and CC 150 demonstrated strong anti-inflammatory properties. According to studies conducted on the biological activities of the various phytochemicals, the flavolignans (Shah *et al.* 2021) and flavones (Aboulaghras *et al.* 2022) glycosides (Khan *et al.* 2020) saponins (Passos *et al.* 2021) and tannins (Soyocak *et al.* 2019) have been shown to induce analgesia and anti-inflammatory effects due to their ability to inhibit key enzymes involved in the inflammation process such as COX-1 (cyclooxygenase 1), COX-2 (cyclooxygenase 2), sPLA2 (secretory phospholipase A2), and 15-LOX-2 (15-lipoxygenase). The extracts and standard drug exhibited their pharmacological activity after 3 hr. This might be explained by the lag-time required for extract and drug to reach the site of action.

Table 3. Anti-Inflammatory Effect of Indomethacin and *C. colocynthis* Extract on Carrageenan Induced Paw Edema

Treatment	Dose (mg/kg)	Degree of Swelling S.E.M	Edema Inhibition (%)
Control	-	1.99 ± 0.010	0
CC	50	1.25 ± 0.022	70.4 ± 3.015
CC	100	1.05 ± 0.021	90.5 ± 2.06
CC	150	1.01 ± 0.023	96.8 ± 2.15
Control	-	1.99 ± 0.010	0
Indomethacin	5	1.30 ± 0.025	69.7 ± 2.51
Indomethacin	10	1.28 ± 0.023	74.5 ± 2.34
Indomethacin	15	1.15 ± 0.020	80.00 ± 2.26

CONCLUSIONS

1. The results suggested that the aqueous seed extract of *Citrullus colocynthis* possesses substantial anti-inflammatory properties. Furthermore, it is essential for the relief of central pain and exhibits strong peripheral antinociceptive activity.
2. The cytotoxic, antibacterial, analgesic, and anti-inflammatory qualities of the extract are probably due to the phytochemical components. *C. colocynthis* extract also was evaluated for its cytotoxicity against malignant cell lines, and the results showed that it was rather effective against all tested Gram-positive bacteria.
3. Using the hot plate approach for analgesia assessment, the best pain alleviation was seen in all experimental groups at the 120-min point. In the carrageenan-induced paw edema model, the variants CC150 and CC200 exhibited strong anti-inflammatory properties, as shown by decreased paw swelling and a greater percentage of edema prevention. Therefore, this study validates the use of *C. colocynthis* aqueous seed extract in folk medicine, supporting its traditional use for the management of pain and inflammation.
4. Future research on *C. colocynthis* aqueous seed extract ought to include the exploration of combination therapies and expanded clinical trials to improve its anti-inflammatory and analgesic effects. It is important to conduct comprehensive toxicological studies to understand the safety of its long-term use.
5. Furthermore, initiatives should prioritise the standardisation and advancement of pharmaceutical formulations to guarantee consistent safety and efficacy. Addressing these areas could reinforce existing discoveries and establish the foundation for novel therapeutic applications.

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Data Availability

The data pertaining to the conclusions of this study can be obtained from the respective authors upon a reasonable request.

Conflict of Interest

All the authors have declared that no conflict of interest related to this work.

REFERENCES CITED

Abbas, D. A. (2013). "Analgesic, anti-inflammatory and antidiarrheal effects of *Datura stramonium* hydroalcoholic leaves extract in mice," *IJRRAS* 14, 193-199.

Aboulaghras, S., Sahib, N., Bakrim, S., Benali, T., Charfi, S., Guaouguaou, F.-E., Omari, N. E., Gallo, M., Montesano, D., Zengin, G., Taghzouti, K., and Bouyahya, A. (2022). "Health benefits and pharmacological aspects of chrysoeriol," *Pharmaceuticals* 15(8), article 973. DOI: 10.3390/ph15080973

Bennett, T. M., Modna, Y., and Shah, D. K. (2024). "The impact of opioid drugs on memory and other cognitive functions: A review," *Journal of Biosciences and Medicines* 12, 4. DOI: 10.4088/PCC.11m01326

Chi, S. C. H., and Jun, H. W. (1990). "Anti-inflammatory activity of ketoprofen gel on carrageenan-induced paw edema in rats," *Journal of Pharmaceutical Sciences* 79, 974-977. DOI: 10.1002/jps.2600791106

Clinkard, D., Buckley, N., Diatchenko, L., and Ghasemlou, N. (2024). "The role of acute inflammation in pain resolution is time to rethink the use of routine anti-inflammatories in surgical practice," *Can. J. Anesth./J. Can. Anesth*. 71, 1349-1352. DOI: 10.1007/s12630-024-02837-8

Gordon, D. B., de Leon-Casasola, O. A., Wu, C. L., Sluka, K. A., Brennan, T. J., and Chou, R. (2016). "Research gaps in practice guidelines for acute postoperative pain management in adults, findings from a review of the evidence for an American pain society clinical practice guideline," *J. Pain* 17(2), 158-166. DOI: 10.1016/j.jpain.2015.10.023

International Association for the Study of Pain (2012). "Unrelieved pain is a major global healthcare problem," *Primary Care Companion CNS Disorders* 14.

Meybodi, M. S. K. (2020). A review on pharmacological activities of Citrullus colocynthis (L.) Schrad. *Asian J Res Rep Endocrinol*, *25*, 34. Article no.AJRRE.57333

Khan, H., Pervaiz, A., Intagliata, S., Das, N., Venkata, K. C. N., Atanasov, A. G., Najda, A., Nabavi, S. M., Wang, D., Pittala, V., and Bishayee, A. (2020). "The analgesic potential of glycosides derived from medicinal plants," *Daru J of Pharmaceutical Science* 28, 387-401. DOI: 10.1007/s40199-019-00319-7

Kim, E., Cui, J., Zhang, G., and Lee, Y. (2022). "Physiological effects of green-colored food-derived bioactive compounds on cardiovascular and metabolic diseases," *Applied Sciences* 12(4), article 1879. DOI: 10.3390/app12041879

Li, Q. Y., Munawar, M., Saeed, M., Shen, J. Q., Khan, M. S., Noreen, S., Alagawany, M., Naveed, M., Madni, A., and Li, C. X. (2022). "*Citrullus colocynthis* (L.) Schrad (bitter apple fruit): Promising traditional uses, pharmacological effects, aspects, and potential applications," *Frontiers in Pharmacology* 12, article 791049. DOI: 10.3389/fphar.2021.791049

Maisetta, G., Batoni, G., Caboni, P., Esin, S., Rinaldi, A.C., and Zucca, P. (2019). "Tannin profile, antioxidant properties, and antimicrobial activity of extracts from two Mediterranean species of parasitic plant *Cytinus*," *BMC Complement Altern. Med.* 19, 82. DOI: 10.1186/s12906-019-2487-7

Mergia, E., Shibeshi, W., Terefe, G., and Teklehaymanot, T. (2016). "Antitrypanosomally activity of *Verbascum sinaiticum* Benth. (Scrophulariaceae) against *Trypanosoma congolense* isolates," *BMC Complementary and Alternative Medicine* 16, 362. DOI: 10.1186/s12906-016-1346-z

Murray, C. B., de la Vega, R., Murphy, L. K., Kashikar-Zuck, S., and Palermo, T. M. (2022). "The prevalence of chronic pain in young adults, a systematic review and meta-analysis," *PAIN* 163(9), e972-e984. DOI: 10.1097/j.pain.0000000000002541

National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2024). *Guide for the Care and Use of Laboratory Animals*, 8th edition, National Academies Press, Washington, D.C., USA. DOI: 10.17226/12910

Olmedo, D. A., Vasquez, Y., Antonio Morán, J., De León, E. G., Caballero-George, C., and Solís, P. N. (2024). "Understanding the *Artemia salina* (brine shrimp) test: Pharmacological significance and global impact," *Combinatorial Chemistry & High Throughput Screening (CCHTS)* 27(4). DOI: 10.2174/1386207326666230703095928

Passos, F. R., Araújo-Filho, H. G., Monteiro, B. S., Shanmugam, S., de Souza Araújo, A. A., Silva Almeida, J. R. D., Thangaraj, P., Quintans Júnior, L. J., and Quintans, J. S. (2021). "Anti-inflammatory and modulatory effects of steroidal saponins and sapogenins on cytokines, A review of pre-clinical research," *Phytomedicine* (96), article 153842. DOI: 10.1016/j.phymed.2021.153842

Rao, V., and Poonia, A. (2023). "*Citrullus colocynthis* (bitter apple): Bioactive compounds, nutritional profile, nutraceutical properties and potential food applications: A review," *Food Prod Process and Nutr.* 5, 4. DOI: 10.1186/s43014-022-00118-9

Rosa, A., Rescigno, A., Piras, A., Atzeri, A., Scano, P., Porcedda, S., Zucca, P., and Assunta Dessì, M. (2012). "Chemical composition and effect on intestinal Caco-2 cell viability and lipid profile of fixed oil from *Cynomorium coccineum* L.," *Food Chem. Toxicol.* 50(10), 3799-3807. DOI: 10.1016/j.fct.2012.07.003

Sakurai, Y. (2015). "Early-stage juvenile fibromyalgia in a 12-year-old girl," *Open Journal of Pediatrics* 5(4). DOI: 10.4236/ojped.2015.54053

Soyocak, A., Kurt, H., Cosan, D. T., Saydam, F., Calis, I. U., Kolac, Uk., Koroglu, ZO., Degirmenci, I., Sahin Mutlu, F., and Gunes, H. V. (2019). "Tannic acid exhibits anti-inflammatory effects on formalin-induced paw edema model of inflammation in rats," *Hum. Exp. Toxicol*. 38, 1296-1301. DOI: 10.1177/0960327119864154

Schofield, P., Dunham, M., Martin, D., Bellamy, G., Francis, S. A., Sookhoo, D., Bonacaro, A., Hamid, E., Chandler, R., Abdulla, A., Cumberbatch, M., & Knaggs, R. (2022). Evidence-based clinical practice guidelines on the management of pain in older people - a summary report. *British journal of pain*, *16*(1), 6–13. DOI: 10.1177/2049463720976155

Shah, M., Jan, H., Drouet, S., Tungmunnithum, D., Shirazi, J. H., Hano, C., and Abbasi, B. H. (2021). "Chitosan elicitation impacts flavonolignan biosynthesis in *Silybum marianum* (L.) Gaertn cell suspension and enhances antioxidant and anti-inflammatory activities of cell extracts," *Molecules* 26(4), article 791. DOI: 10.3390/molecules26040791

Zhang, Y. H., Adamo, D., Liu, H., Wang, Q., Wu, W., Zheng, Y. L., and Wang, X. Q. (2023). "Inflammatory pain: Mechanisms, assessment, and intervention," *Frontiers in Molecular Neuroscience* 16, article 1286215. DOI: 10.3389/fnmol.2023.1286215

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